

MICROSOME VACCINE

5 The present invention relates to a novel peptide-based vaccines, uses of such vaccines in prophylactic and therapeutic treatment of human and animal diseases, such as viral infection and cancer.

10 Most of the successful vaccines depend on neutralising antibodies raised by classic attenuated or killed pathogens. However, for pathogens causing chronic infection- such as HIV, hepatitis C virus, mycobacteria and parasites – or in the case of cancer, a T-cell mediated immune response is crucial. Molecular understanding of MHC antigen presentation and the T-cell immune responses led to the use of defined antigenic peptide plus cytokines and/or co-stimulatory molecules in attempts to develop vaccines. One of the basic problems in all these attempts was the difficulty to reconstitute an antigen delivery system that is qualitatively and quantitatively similar to antigen presenting cells (APC) *in vivo*.

20 CD8+ cytotoxic T lymphocytes (CTL) recognise antigens as small antigenic peptides that assemble with major histocompatibility complex (MHC) class I molecules. The antigenic peptides are generated in the cytosol of APC and subsequently translocated into the lumen of the endoplasmic reticulum (ER) (Rock, K. L. & Goldberg, A. L. *Annu Rev Immunol* 17, 739-779 (1999)). The MHC class I heavy chain is synthesised and inserted into the lumen of the ER and where it forms a dimer with b2-microglobulin (b2M) (Natarajan *et al Rev Immunogenet* 1, 32-46 (1999); Pamer E, & Cresswell P, *Annu Rev Immunol*. 16 323-358 (1998)). The dimers are retained in the ER until they assemble with proper antigenic peptides. The process of MHC class I dimer and assembly with peptides in the ER is catalysed by chaperones such as BIP, calnexin, calreticulin, and Erp57 (Paulsson K, & Wang P., *Biochim Biophys Acta*. 1641(1) 1-12 (2003)).

30 The assembled MHC class I are rapidly expressed on the cell surface of APC, such as infected or malignant cells. The recognition of peptide-MHC class I by T cell receptor leads the CTL to kill target cells expressing infectious or tumor antigens.

Following the identification of CTL recognized epitopes from viral or cancer proteins, synthetic peptide-based vaccines designed to elicit T-cell immunity became an attractive approach to the prevention or treatment of infectious and malignant diseases (Furman MH, & Ploegh HL., *J Clin Invest.* 110 (7) 875-9 (2002); Berinstein N. *Semin Oncol.* 30 (3) (Suppl 8), 1-8 (2003); Falk *et al Nature* 348, 248-251. (1990); (Van Bleek GM, & Nathenson SG., *Nature* 348: 213-216 (1990); Kast, W.M., & Melief, C.J. *Immunol. Lett.* 30:229-232 (1991)). There are a number of different forms of peptide vaccines based on these delivery systems. The simplest form is peptides dissolved in aqueous solutions. Direct injection of soluble antigenic peptides was shown to be unsuccessful at stimulating CTL responses, either because of their rapid biodegradation or induction of T cell anergy resulting from the antigenic stimulation by immature APC (Kyburz, D. *et al. Eur. J. Immunol.* 23:1956-1962 (1993); Toes, R.E *et al Proc. Natl. Acad. Sci. USA.* 93:7855-7860 (1996); Amoscato *et al J. Immunol.* 161, 4023-4032 (1998)). An additional complication reported from the use of synthetic peptide-derived vaccines is the induction of CTLs that, while they are capable of killing target cells that are exogenously pulsed with peptide, they are not able to recognise target cells that naturally process and present the peptide epitope, such as infected or malignant cells (Dutoit, V. *et al. J. Clin. Invest.* 110:1813-1822 (2002)).

It has been reported that MHC class I antigen presentation is qualitatively controlled in the ER for selecting correct peptides. Only the correctly assembled MHC class I could express on the surface of APC. The use of adjuvants did little to increase the presentation quality of synthetic peptides (Schijns, V.E. 2001. *Crit. Rev. Immunol.* 21:75-85 (2001). An improved version of the peptide-vaccine has been constructed as an artificial lipo-membrane (BenMohamed *et al Lancet Infect Dis.* 2(7), 425-31 (2002)) with peptide-loaded recombinant MHC class I. Although liposome strategy is able to incorporate peptide bound MHC class I molecules in the lipid membrane before injection into patients, the sophisticated loading system in the ER of APC could not be easily imitated by a simple mixture of recombinant MHC class I, synthetic peptide and liposomes. Only a few peptides would assemble with

recombinant MHC class I *in vitro* (Ostergaard Pedersen L, *et al Eur J Immunol.* 31(10), 2986-96 (2001).

5 In addition, the incorrect orientation of inserted MHC class I and lack of co-stimulatory molecules made it difficult to induce effective immune responses. Since the professional APCs have the unique ability of presenting optimal antigen and for initiating a cellular immune response by naïve T cells, strategies are being developed to generate autologous dendritic cells (DC), a key APC, as vaccine vehicles *ex vivo* (Banchereau, J. *et al. Annu. Rev. Immunol.* 18:767-811 (2000)). Initial studies showed
10 that antigenic peptide-pulsed DC used as vaccines *in vivo* could induce a CTL response (Tsai, V. *et al. J. Immunol.* 158:1796-1802 (1997)). Despite the positive evidence reported from a number of human clinical trials, there is no biochemical evidence showing that the pulsed peptides are indeed loaded on the surface MHC class I, which questions the efficiency of peptide-pulsed APCs to induce effective
15 immune responses.

There is therefore a need for a vaccine preparation that can overcome these problems and present a therapeutically effective alternative to conventional vaccines. Such vaccines should achieve the quality of the endogenous presented antigen by APC cells
20 while preserving high efficacy and avoiding side effects.

According to a first aspect of the invention, there is provided a vaccine composition comprising isolated inverted microsomes from an animal cell, or membrane fragments thereof, in association with an externally disposed peptide antigen and a protein of the
25 Major Histocompatibility Complex (MHC).

The microsomes of the present invention are derived from an animal cell and may therefore arise from the following compartments present in a eukaryotic cell: endoplasmic reticulum, lysosome; endosome, or components of the endocytic
30 pathway.

The microsome may be isolated with a protein of the MHC already present in the membrane of the microsome or of the fragment. Alternatively, the MHC protein can be introduced into the microsome or fragment subsequently. The ER derived microsomes contain both MHC class I and class II molecules (Bryant *et al Adv Immunol.* **80**, 71-114 (2002)).

The present invention is equally applicable with respect to the MHC class I restricted antigenic peptides as well as the MHC class II molecules. The protein of the MHC in the composition may be from a heterologous source with respect to the cell from which the microsomes are obtained.

The MHC family of proteins are encoded by the clustered genes of the major histocompatibility complex (MHC). MHC molecules are expressed on the cells of all higher vertebrates. They were first demonstrated in mice and called *H-2 antigens* (histocompatibility-2 antigens). In humans they are called *HLA antigens* (human-leucocyte-associated antigens) because they were first demonstrated on leucocytes (white blood cells). Class I and class II MHC molecules are the most polymorphic proteins known - that is, they show the greatest genetic variability from one individual to another - and they play a crucial role in presenting foreign protein antigens to cytotoxic and helper T cells, respectively. Whereas class I molecules are expressed on almost all vertebrate cells, class II molecules are restricted to a few cell types that interact with helper T cells, such as B lymphocytes and macrophages. Both classes of MHC molecules have immunoglobulin-like domains and a single peptide-binding groove, which binds small peptide fragments derived from foreign proteins. Each MHC molecule can bind a large and characteristic set of peptides, which are produced intracellularly by protein degradation. After they form inside the target cell, the peptide-MHC complexes are transported to the cell surface, where they are recognized by T cell receptors. In addition to their antigen-specific receptors that recognize peptide-MHC complexes on the surface of target cells, T cells express CD4 or CD8 co-receptors, which recognize non-polymorphic regions of MHC molecules on the target cell: helper cells express CD4, which recognizes class II MHC molecules, while cytotoxic T cells express CD8, which recognizes class I MHC

molecules. (Alberts *et al*, "*Molecular Biology of the Cell*", 3rd edition, 1229-1235 (1994)).

5 The MHC class I consists of heavy chain and Beta-2-microglobulin. Human MHC class I heavy chains are encoded by three separate genetic loci called HLA A, B, C. They are noncovalently associated with a small protein called beta-2-microglobulin. An example of a human MHC class I protein is HLA class I histocompatibility antigen, A-2 alpha chain precursor (MHC class I antigen A*2) is shown in Figure 13 (database accession no. P01892); or HLA class I histocompatibility antigen, B-7 alpha chain precursor (MHC class I antigen B*7) as shown in Figure 13 (database accession
10 no. P01889).

MHC class II are composed of two noncovalently bonded chains an α -chain and an β -chain. Both chains are coded by genes in I-region associated (Ia) antigens. Examples
15 of such proteins are HLA class II histocompatibility antigen, DRB3-1 beta chain precursor (MHC class I antigen DRB3*1) shown in Figure 14 (database accession no. P79483); and MHC class II histocompatibility antigen HLA-DQ alpha 1 (DQw4 specificity) precursor, also shown in Figure 14 (database accession A37044).

20 The sequences of the MHC class I and II cDNAs and genomic DNAs are published and available (www.ncbi.nlm.nih.gov/Genbank).

All eucaryotic cells have an endoplasmic reticulum (ER). Its membrane typically constitutes more than half of the total membrane of an average animal cell. It is
25 organized into a netlike labyrinth of branching tubules and flattened sacs extending throughout the cytosol. The tubules and sacs are all thought to interconnect, so that the ER membrane forms a continuous sheet enclosing a single internal space. This highly convoluted space is called the ER lumen or the *ER cisternal space*, and it often occupies more than 10% of the total cell volume. The ER membrane separates the ER
30 lumen from the cytosol, and it mediates the selective transfer of molecules between these two compartments.

The ER plays a central part in lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles, including the ER itself, the Golgi apparatus, lysosomes, endosomes, secretory vesicles, and the plasma membrane. The ER membrane also makes a major contribution to mitochondrial and peroxisomal membranes by producing most of their lipids. In addition, almost all of the proteins that will be secreted to the cell exterior - as well as those destined for the lumen of the ER, Golgi apparatus, or lysosomes - are initially delivered to the ER lumen (Alberts *et al*, "*Molecular Biology of the Cell*", 3rd edition, 577-595 (1994)).

10

The lysosome is a specialised organelle containing specialised enzymes for the degradation of internal cellular proteins that are required to be destroyed, or for the destruction of external foreign proteins or parasites that have been targeted for destruction by the immune system.

15

The endosome is a cell organelle that forms part of the endocytic pathway in the cell. There is a constant flow of endocytic vesicles that flow from the cell surface to the endosome or to the lysosome. The vesicles form by a process of "budding-off" from the external plasma membrane, known as invagination, or the vesicles can form from the internal cell organelles to which they ultimately return. Endocytosis is the process by which a cell internalises external receptors with or without bound ligand and also one way by which the cell can sample its external environment.

20

Compositions in accordance with the present invention may be optionally formulated with an appropriate adjuvant, and/or cytokines that promote T-cell responses, such as an interferon or an interleukin, e.g. IL-2, IL-15, IL-6, GM-CSF, IFN γ , other cytokines promoting T-cell responses, and/or conventional adjuvant. These can be suitably mixed with the microsomes loaded with antigen prior to administration, or may be suitably prepared as membrane-bound constituents of the microsomes.

25

30

Microsomes in the context of the present invention are the cell free membrane vesicles of the endoplasmic reticulum (ER), lysosomal, or endosomal compartments

of any animal cell able to present antigenic peptide by means of the Major Histocompatibility Complex (MHC). The definition of ER-derived microsomes is based on the presence of so-called "ER-markers" which are proteins normally resident in the ER, such as BIP, p58, calnexin, calreticulin, tapasin. The definition of a
5 lysosomal-derived microsome is based on the presence of the specific markers LAMP1 and/or LAMP2. Microsomes are recognised as such by their morphology as seen under the electron microscope following preparation from an animal cell.

The microsomes contained in a composition of the present invention can be isolated
10 by any convenient means. Suitable methods include those of Saraste *et al* and/or Knipe *et al* (Saraste *et al Proc. Natl. Acad. Sci. U. S. A.* **83**, 6425-6429 (1986) and Knipe *et al J. Virol.* **21**, 1128-1139 (1977)). Such methods comprise homogenisation of cells or tissues, followed by separation of the cell nucleus by centrifugation at 7500rpm for 10 minutes, then recovering the "rough" microsomes by centrifugation at
15 15500 rpm for 54 minutes. "Rough" microsomes are microsomes that have ribosomes attached. The resuspended "rough" microsomes are then further purified by centrifugation through a sucrose cushion for differential centrifugation at 110,000g for 60 minutes. The rough microsomes were subfractionated by further centrifugation at 37,000rpm for 10 hours on a sucrose gradient (to reach isopyknic conditions), and
20 the ER containing fractions determined by Western blotting with appropriate antibody, for example anti-p58 antibody.

Inverted microsomes are the result of further processing, e.g. repeated freeze-thaw process steps, carried out on isolated microsomes which causes the disruption and
25 reformation of the external membrane of the microsome such that the "inside" face of the membrane is presented on the "outside" of the inverted microsome. The microsomes that result from such processing are therefore described as "inside-out" or "inverted" microsomes. The process of preparing the "inside-out" or inverted microsomes results in the absence of the lumen structure seen in ordinary microsome
30 preparations.

In compositions according to the present invention, the microsome may comprise a membrane fragment thereof. Suitably, such membrane fragments may be prepared by the method comprising the use of detergents or repeated freeze-thawing or sonication to break the microsome structure. Such fragments may also be similarly loaded with peptide antigen to form a composition of the present invention. Preferably, the membrane fragments are derived from intracellular membranes with markers specific to the ER or to the lysosomes.

In a vaccine composition of the present invention, there may also be a percentage of microsomes with a non-inverted structure, i.e. a membrane orientation that corresponds to the *in situ* arrangement after standard microsome preparation with an "inside" corresponding to the lumen of the ER, endosome or lysosome prior to microsome preparation. However, at least about 75% to about 95%, suitably at least about 90% of microsomes in the vaccine compositions of the invention have a reversed membrane orientation to *in situ* microsomes and are therefore described as being "inside-out" or inverted microsomes. The compositions may therefore additionally comprise a percentage of non-inverted microsomes.

In other embodiments of the invention, the composition may be more homogenous, and so may comprise at least about 95%, 96%, 97%, 98%, 99% or 100% of microsomes having an inverted or reversed (or "inside-out") membrane orientation compared to microsomes prepared from cells without further processing.

The microsomes may be loaded with antigen first and then subjected to further processing so as to provide inverted or "inside-out" microsomes thus exposing the inner surface of the ER membrane, or the microsomes can be prepared from a cell source where the preferred antigen peptide is already present in the microsomes, or the microsomes may be processed to provide inverted or "inside-out" microsomes first and then subsequently loaded with antigen.

Lysosomes and endosomes can be prepared by an equivalent procedure. Lysosomal microsomes which are purified from the endocytotic compartment of the animal cell

include both lysosomes and endosomes. After fractionation of the total cellular membranes in the purification procedure for the preparation of ER-derived microsomes, the lysosomal membranes are defined by antibodies to its markers LAMP1 and LAMP2.

5

The purified lysosomal microsomes are then processed to yield inverted or "inside-out" microsomes or membrane fragments as described above which, if necessary, can then be loaded with MHC restricted peptides under acid conditions, such as for example at a pH of less than pH3, preferably from pH 3 to pH 3, suitably at around pH 2.5.

10

The animal cell from which the isolated microsome population is to be prepared can be any generally convenient cell type that has MHC molecules expressed by the cell. For example, cells of the blood or of the immune system such as, B-cells and macrophages, the so-called antigen presenting cells (APCs). However, cell types could also be used from tissues such as liver, kidney, lung, brain, heart, skin, bone marrow, pancreas etc.

15

The cells may be of a human or of a non-human animal. Suitably, the animal is a mammal. The animal may be a rodent species, e.g. a mouse, a rat or a guinea pig, or another species such as rabbit, or a canine or feline, or an ungulate species such as ovine, porcine, equine, caprine, bovine, or a non-mammalian animal species, e.g. an avian (such as poultry, e.g. chicken or turkey).

20

The cells from which the microsomes are prepared may be a cell line in culture. The cell line may be an immortalised cell line. The cell line may be ultimately derived from a non-embryonic tissue source.

25

In certain embodiments of the invention, the source of cells may be a genetically modified source of animal cells, such as a cell line, or a transgenic non-human animal. The cells or tissue from which the microsomes are prepared may be a humanised

30

animal tissue or cell from a transgenic non-human animal whose genome has been modified by the insertion of one or more human genes.

5 In embodiments of the invention relating to microsomes prepared from a transgenic non-human animal or transgenic cell line, the transgenesis is the introduction of an additional gene or genes or protein-encoding nucleic acid sequence or sequences. The transgene may be a heterologous gene or an additional copy of a homologous gene, optionally under the control of a constitutive promoter or an inducible promoter. The transgenesis may be transient or stable transfection of a cell or a cell line, or an
10 episomal expression system in a cell or a cell line.

However, it is in the field of human medicine, in which the compositions of this aspect of the invention are expected to find greatest application as vaccines. It is therefore preferred that the source of cells from which the microsomes are prepared
15 has an MHC allotype that is compatible to the MHC of the recipient of the composition when used as a vaccine.

In one embodiment according to this aspect of the invention, the source of cells from which the microsomes are prepared may be the ultimate recipient of the composition
20 when used as a vaccine.

Alternatively, a suitable source of human cells may be from a cell line, for example a non-embryo derived cell-line, suitably a B-cell line such as cell line 221. Such cell lines may also advantageously not express proteins of the Major Histocompatibility
25 Complex (MHC) type class I and/or class II. This embodiment of the invention may be a more preferred embodiment for the manufacture of vaccines on a commercial scale, where non-individual vaccines are produced from such cell lines which have been engineered with different MHC allotypes.

30 Cell line 221 is an example of such a MHC negative cell line. The absence of a native MHC class I expression in such cells permits the modification of the cell line to express MHC class I of any desired genotype. This may be particularly important in

achieving the full immunising effects of the vaccine composition, since different human populations express different MHC proteins. In such compositions of the invention, the MHC protein may therefore be of a heterologous source with respect to the cell from which the microsomes are obtained.

5

Some of the MHC class I, like HLA A2, are expressed in more than 20% of the population. In circumstances where a MHC negative cell line is used, one or more than one compatible MHC gene is transfected into the cell line by means of conventional gene transfer methods and the transgene is constructed into a expression vector. The expression cassette of expression construct normally includes standard promoter, such as CMV promoter, or elongation factor I promoter or actin promoter, enhancer, inserted transgene and the poly-A signal to achieve optimal expression. Before transfection, the expression cassette will be isolated from the plasmid backbone to avoid the expression of bacterial plasmid genes in transfected cells. The sequences of the MHC class I and II cDNAs and genomic DNAs are published and available (www.ncbi.nlm.nih.gov/Genbank). A MHC class I transfectants Bank can be constructed by using MHC class I negative or selected MHC class positive cell lines to transfect most of the MHC class I genes, respectively. The selection of the expression cassette will be dependent on the optimal expression of the transgene.

20

Transfection of the antigen presenting cells may be achieved using standard recombinant techniques, e.g. using a suitable vector comprising a nucleic acid sequence encoding a MHC protein of interest. The term "vector" generally refers to any nucleic acid vector which may be RNA, DNA or cDNA. The vector can be described alternatively as an "expression vector".

25

The terms "vector" or "expression vector" may include, among others, chromosomal, episomal, and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof,

30

such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Generally, any vector suitable to maintain, propagate or express nucleic acid to express a polypeptide in a host may be used for expression in this regard. The vector may be constructed from a bacterial plasmid, for example the
5 bacterial plasmid pUC18.

The vector may provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Preferred among inducible vectors are vectors that can be induced for
10 expression by environmental factors that are easy to manipulate, such as temperature, nutrient additives, hypoxia and/or the presence of cytokines or other biologically active factors. Particularly preferred among inducible vectors are vectors that can be induced for expression by changes in the levels of chemicals, for example, chemical additives such as antibiotics. A variety of vectors suitable for use in the invention,
15 including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those skilled in the art.

Recombinant expression vectors will include, for example, origins of replication, a promoter preferably derived from a highly expressed gene to direct transcription of a
20 structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector.

Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites,
25 polyadenylation regions, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking non-transcribed sequences that are necessary for expression. Preferred mammalian expression vectors according to the present invention may be devoid of enhancer elements.

30 The promoter sequence may be any suitable known promoter, for example the human cytomegalovirus (CMV) promoter, the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters or the promoters of

retroviral LTR's, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter. The promoter may comprise the minimum sequence required for promoter activity (such as a TATA box without enhancer elements), for example, the minimal sequence of the
5 CMV promoter (mCMV). Preferably the promoter is a mammalian promoter that can function at a low basal level devoid of an enhancer element.

Preferably, the promoter is contiguous to the nucleic acid sequence encoding the MHC protein to be transfected into the antigen presenting cell. It is contemplated that
10 variants, for example, homologues or orthologues, of the promoters described herein are part of the present invention.

The backbone of the expression vector of the first aspect of the invention may be derived from a vector devoid of its own promoter and enhancer elements, for example
15 the plasmid vector pGL2. Enhancers are able to bind to promoter regions situated several thousands of bases away through DNA folding (Rippe *et al TIBS* 1995; 20: 500-506 (1995)).

The expression vectors may also include selectable markers, such as antibiotic
20 resistance, which enable the vectors to be propagated.

The nucleic acid sequences of the vector containing nucleic acid encoding the MHC protein to be transfected may encode a reporter protein as described above, such as a chloramphenicol acetyl transferase ("CAT") transcription unit, luciferase or green
25 fluorescent protein (GFP). The application of reporter genes relates to the phenotype of these genes which can be assayed in a transformed cell and which is used, for example, to analyse the induction and/or repression of gene expression. Reporter genes for use in studies of gene regulation include other well known reporter genes including the *lux* gene encoding luciferase which can be assayed by a
30 bioluminescence assay, the *uidA* gene encoding β -glucuronidase which can be assayed by a histochemical test, the *lacZ* gene encoding β -galactosidase which can be

assayed by a histochemical test, the enhanced green fluorescent protein which can be detected by UV light, UV microscopy or by FACS.

5 The DNA comprising the nucleic acid sequence of the MHC protein may be single or double stranded. Single stranded DNA may be the coding or sense strand, or it may be the non-coding or anti-sense strand. For therapeutic use, the nucleic acid sequences are in a form capable of being expressed in the subject to be treated.

10 The termination sequences in the vector may be a sequence of adenylate nucleotides which encode a polyadenylation signal. Typically, the polyadenylation signal is recognisable in the subject to be treated, such as, for example, the corresponding sequences from viruses such as, for human treatment, the SV40 virus. Other termination signals are well known in the art and may be used.

15 Preferably, the polyadenylation signal is a bidirectional terminator of RNA transcription. The termination signal may be the polyadenylation signal of the simian 40 virus (SV40), for example the SV40 late poly(A). Alternatively, the termination sequence may be the polyadenylation signal of bovine growth hormone which results in maximal expression when combined with a CMV promoter (Yew *et al. Human*
20 *Gene Therapy*, 8: 575-584 (1997)).

In addition the expression vector may comprise a further polyadenylation sequence, for example an SV40 early poly(A). Such a further poly (A) may be located upstream of the nucleic acid sequence encoding the MHC protein to reduce cryptic transcription
25 which may have initiated within the vector thereby ensuring that basal gene expression from the vector is minimal.

Gene expression from integrated viral genomes may be susceptible to chromosomal positional effects. Such effects include transcriptional silencing and promoter
30 activation by nearby heterologous enhancers. In addition, integrated sequences can activate expression of nearby genes and oncogenes. These effects are reduced through the use of elements which form boundaries to the inserted viral genome.

Insulators are genetic elements such as the chicken β -globin 5' DNase I hypersensitive site (5'HS4) which mark a boundary between an open chromatin domain and a region of constitutively condensed chromatin.

5 Other elements termed scaffold or matrix attachment regions (S/MAR) anchor chromatin to nuclear structures and form chromosomal loops which may have a physiological role in bringing distal regulatory elements into close proximity to a corresponding promoter. An example is located in the human interferon- γ locus and is termed the IFN-SAR. Both insulators and S/MAR can reduce position effects with
10 greatest activity demonstrated when they were combined in a lentiviral vector (Ramezani *et al*, *Blood* 101: 4717-24, (2003)). Clearly such elements can be of benefit in regulated vectors such as those described herein after they are integrated into the host cell genome.

15 The compositions of the present invention comprise a microsome, or a fragment thereof, in association with an externally disposed peptide antigen that has been loaded into the microsome. The association may be such that the peptide antigen is inserted in the membrane of the microsome such at least one epitope of the peptide
20 antigen is exposed with respect to the outer membrane of the microsome. The membrane of microsomes further contains a protein of the MHC that presents the peptide antigen to T-cells in order for the antigen to be recognised by the immune system. The MHC protein is either naturally present in the cell organelles of the cell from which the microsomes were produced, or it is a MHC protein that has been
25 transfected into the cell through recombinant DNA techniques and expressed, prior to preparation of the microsomes. The inserted antigenic peptide and the MHC protein form an association in the membrane of the microsome which permits external disposition of the proteins for interaction with the cells of the immune system.

30 The antigenic peptides may be introduced or loaded into the microsome by means of incubating the microsome with the peptide antigen in the presence of a nucleoside triphosphate (NTP), for example adenosine triphosphate (ATP) and NTP re-

generation system. It appears that an NTP, such as ATP, facilitates the incorporation of the peptide antigen into the microsome through protein transporters located in the membrane of the microsome. Without wishing to be bound unnecessarily by theory, it appears that once the microsome is incubated with the peptide antigen in the presence of an NTP that the antigen is able to associate with MHC class I proteins already present in the membrane of the microsome. Alternatively, the antigenic peptides may also loaded into the microsomes after inside-out processing and in this case, the NTP is not required.

The antigenic peptide present in association with the microsome suitably has one or more epitopes. An epitope is the smallest part of an antigen recognisable by the combining site of an immunoglobulin and may be linear or discontinuous. Therefore, any type of MHC binding peptides, natural or synthesized or artificially modified, is included.

The antigenic peptides may be from a source that is foreign, i.e. non-self, or self, i.e. an autoantigen. Foreign antigenic peptides may originate from virus, bacteria, yeast, fungi, protozoa, or other micro-organism (i.e. an infectious agent), or of higher life forms such as plants or animals. In some embodiments of the invention, the antigen may be an auto-antigen, for example an antigen expressed by a neoplastic cell or cell of a cancer tumour, a normal self-protein (in the case of an tolerising vaccine of the invention for an auto-immune disorder).

Where the antigen is from a neoplastic cell or cell of a cancer tumour, the cell may be from a melanoma, lung adenocarcinoma, colon cancer, breast cancer or leukemia cell. Auto-immune disorders include, but are not limited to, Multiple Sclerosis (MS), Systemic Lupus Erythamatosus, Type-1 or Insulin-dependent Diabetes, Antiphospholipid Syndrome, Myasthenia Gravis, Myositis, Sjogren's Syndrome and Rheumatoid arthritis.

In some embodiments of the invention, it may be preferred to prepare the composition with an antigenic peptide of more than one type, or antigenic peptides having a

sequence modified to increase immunogenicity. The cell may also be transfected prior to the preparation of the microsomes with more than one type of MHC molecules which may be useful in the case of recipients of the compositions when used as vaccines who have more than one type of MHC allotype.

5

In a preferred embodiment of this aspect of the invention, there is provided a composition as defined above in which the ratio of antigen to MHC molecule in the microsome is optimal for the induction of a specific immunoresponse, for example in the range of from 0.1 to 1.5, preferably of from 0.2 to 1.2 or 0.5 to 1.0, and most preferably from 0.2-0.5 to 1.0. The amount of loaded antigenic peptides may be different according to the level of immune response induced.

10

Defined antigenic peptides of major diseases can be readily selected from the scientific literature or identified by bioinformatic tools, (Renkvist *et al Cancer Immunol Immunother* **50**, 3-15 (2001); Coulie *et al Immunol Rev* **188**, 33-42 (2002); De Groot *et al Vaccine* **19** (31), 4385-95 (2001)).

15

For example, the influenza virus derived peptides SIINFEKL and ASNENMETM, or the peptide YLQLVFGIEV from melanoma cells.

20

Table 1 shows details of Class I HLA-restricted cancer/testis antigens; Table 2 shows Class I HLA-restricted melanocyte differentiation antigens; Table 3 shows Class I HLA-restricted widely expressed antigens; Table 4 shows Class I HLA-restricted tumor specific antigens; Table 5 shows Class II HLA-restricted antigens; Table 6 shows epitopes derived from fusion proteins; and Table 7 shows frequency of epitopes recognised by a given HLA allele.

25

Further examples are shown in Table 8 of Hepatitis C virus (HCV) peptides from Anthony *et al Clinical Immunol.*, vol. 103, pages 264-276 (2002); in Table 9 of Human Immunodeficiency Virus-1 (HIV-1) from Kaul *et al J. Clinical Invest.*, vol. 107, pages 1303-1310 (2001); in Table 10 of Hepatitis C Virus (HCV) peptides from

30

Koziel *et al J. Virol.*, vol. 67, pages 7522-7532 (1993); and in Table 11 of Hepatitis C Virus (HCV) from He *et al PNAS USA*, vol. 96, pages 5692-5697 (1999).

5 The antigenic peptide epitopes may be present as a monomer or as repeated sequence of the epitope, such as dimer, trimer, tetramer, or higher multiple, such as a pentamer, hexamer, heptamer, octamer, nonamer or decamer. Fragments of the epitope sequences can be used, as well as overlapping sequences that include the epitope sequence.

10 The term "peptide" includes both polypeptide and protein, unless the context specifies otherwise.

Such peptides include analogues, homologues, orthologues, isoforms, derivatives, fusion proteins and proteins with a similar structure or are a related polypeptide as
15 herein defined.

The term "analogue" as used herein refers to a peptide that possesses a similar or identical function as a protein sequence described herein but need not necessarily comprise an amino acid sequence that is similar or identical to such an amino acid
20 sequence, or possess a structure that is similar or identical to that of a protein described herein. An amino acid sequence of a peptide is "similar" to that of a peptide described herein if it satisfies at least one of the following criteria: (a) the peptide has an amino acid sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%,
25 at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the amino acid sequence of a peptide described herein; (b) the peptide is encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding at least 5 amino acid residues (more preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid
30 residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid

residues, at least 125 amino acid residues, or at least 150 amino acid residues) of a peptide sequence described herein; or (c) the peptide is encoded by a nucleotide sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%,
5 at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the nucleotide sequence encoding a peptide described herein.

Stringent conditions of hybridisation may be characterised by low salt concentrations or high temperature conditions. For example, highly stringent conditions can be
10 defined as being hybridisation to DNA bound to a solid support in 0.5M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at 65°C, and washing in 0.1xSSC/0.1%SDS at 68°C (Ausubel *et al* eds. "Current Protocols in Molecular Biology" 1, page 2.10.3, published by Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, (1989)). In some circumstances less stringent conditions may be
15 required. As used in the present application, moderately stringent conditions can be defined as comprising washing in 0.2xSSC/0.1%SDS at 42°C (Ausubel *et al* (1989) *supra*). Hybridisation can also be made more stringent by the addition of increasing amounts of formamide to destabilise the hybrid nucleic acid duplex. Thus particular hybridisation conditions can readily be manipulated, and will generally be selected
20 according to the desired results. In general, convenient hybridisation temperatures in the presence of 50% formamide are 42°C for a probe which is 95 to 100% homologous to the target DNA, 37°C for 90 to 95% homology, and 32°C for 70 to 90% homology.

25 A peptide with "similar structure" to that of a peptide described herein refers to a peptide that has a similar secondary, tertiary or quaternary structure as that of a peptide described herein. The structure of a peptide can determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

30 The term "fusion protein" as used herein refers to a peptide that comprises (i) an amino acid sequence of a peptide described herein, a fragment thereof, a related

peptide or a fragment thereof and (ii) an amino acid sequence of a heterologous peptide (*i.e.*, not a peptide sequence described herein).

5 The term "homologue" as used herein refers to a peptide that comprises an amino acid sequence similar to that of a peptide described herein but does not necessarily possess a similar or identical function.

10 The term "orthologue" as used herein refers to a non-human peptide that (i) comprises an amino acid sequence similar to that of a peptide described herein and (ii) possesses a similar or identical function.

The term "related peptide" as used herein refers to a homologue, an analogue, an isoform of , an orthologue, or any combination thereof of a peptide described herein.

15 The term "derivative" as used herein refers to a peptide that comprises an amino acid sequence of a peptide described herein which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The derivative peptide possess a similar or identical function as peptides described herein.

20 The term "fragment" as used herein refers to a peptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at
25 least 90 amino acid residues, at least 100 amino acid residues) of the amino acid sequence of a peptide as described herein, *mutatis mutandis*. The fragment of may or may not possess a functional activity of such peptides.

30 The term "isoform" as used herein refers to variants of a peptide that are encoded by the same gene, but that differ in their isoelectric point (pI) or molecular weight (MW), or both. Such isoforms can differ in their amino acid composition (*e.g.* as a result of alternative splicing or limited proteolysis) and in addition, or in the alternative, may

arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation). As used herein, the term "isoform" also refers to a peptide that exists in only a single form, *i.e.*, it is not expressed as several variants.

5 The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences which results in the highest
10 percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (*i.e.*, % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished
15 using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci. USA* (1990) 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. The NBLAST and XBLAST programs of Altschul et al, *J. Mol. Biol.* (1990) 215:403-410 have
20 incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules encoding a peptide sequence as described herein. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a peptide as
25 described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilised as described in Altschul et al, *Nucleic Acids Res.* (1997) 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilising BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective
30 programs (*e.g.*, XBLAST and NBLAST) can be used. See, for example, <http://www.ncbi.nlm.nih.gov>.

Another example of a mathematical algorithm utilised for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti *Comput. Appl. Biosci.* (1994) 10:3-5; and FASTA described in Pearson and Lipman *Proc. Natl. Acad. Sci. USA* (1988) 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a substance can often be substituted by one or more other such amino acids without eliminating a desired activity of that substance. Thus the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains). Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

Amino acid deletions or insertions may also be made relative to the amino acid sequence of a peptide sequence as described herein. Thus, for example, amino acids which do not have a substantial effect on the activity of such peptides, or at least which do not eliminate such activity, may be deleted. Amino acid insertions relative to the sequence of peptides as described herein can also be made. This may be done to alter the properties of a protein of the present invention (e.g. to assist in identification, purification or expression, where the protein is obtained from a recombinant source,

including a fusion protein. Such amino acid changes relative to the sequence of a peptide from a recombinant source can be made using any suitable technique e.g. by using site-directed mutagenesis. The molecule may, of course, be prepared by standard chemical synthetic techniques, e.g. solid phase peptide synthesis, or by available
5 biochemical techniques.

It should be appreciated that amino acid substitutions or insertions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids. Whether or not natural or synthetic amino acids are used, it is preferred that
10 only L-amino acids are present.

According to the present invention, purified microsomes representing the endoplasmic reticulum, lysosomes or endosomes in antigen-presenting cells (APC) can be used to load antigenic peptides on its MHC class I or II molecules. Results from *in vitro* and
15 *in vivo* immunisation described herein show that peptide-loaded microsome elicits much stronger responses than peptide-loaded APC measured by T cell proliferation and production of IL-2. By quantitating the amount of peptide-receptive MHC class I molecules, the receptive class I molecules on APC surface are below the radio-chemical detection limit. However, a significant amount of peptide bound MHC class
20 I is detected in the microsome. In addition, a similar amount of co-stimulatory molecules, B7.1 and B7.2 is detected in microsomes in comparison to cell surface. Thus, the microsomes loaded with antigenic peptides represent an effective vaccine composition.

25 The present invention has found that more than 50% of the MHC class I molecules in the ER of APC are peptide receptive. By the process of "inside-out", the microsomes loaded with Kb specific OVA-peptide can induce T cell responses *in vitro* and *in vivo*. In contrast, the APCs pulsed with same peptide have much less ability to stimulate T cell responses. Given that the microsomes contain co-stimulatory molecules, the
30 microsomes isolated from APCs represent promising vehicles for peptide vaccines in the future for a wide variety of diseases.

In addition to transfecting selected MHC genes into the animal cell prior to microsome preparation, it may also be desirable to transfect or co-transfect the cells with genes encoding co-stimulatory molecules such as B7 and/or the genes encoding cytokines, for example an interleukin or an interferon, such as IL-2. In the case of
5 cytokines, the transgene will be fused with trans-membrane domain of CD2 or CD4 for targeting the cytokines into the ER membrane. In addition, in order to enrich the level of MHC, co-stimulatory molecules and membrane-bound cytokines in the ER, KDEL or other ER retention signalling (Nilsson T, & Warren G., *Curr Opin Cell Biol.* 6 (4), 517-21 (1994)) will be tagged at the C-terminus of the transgenes for the
10 retention of transgene products in the ER. The expression cassettes for these transgenes are similar to MHC class I transgenes.

According to the present invention, therefore, the vaccine compositions may be co-administered with one or more cytokines, such as an interferon or an interleukin, that
15 can promote T cell immune response such as IL-2, IL-15, IL-6, GM-CSF, IFN γ , other cytokines promoting T cell responses, and/or conventional adjuvant. These can be suitably mixed with the microsomes loaded with antigen prior to administration, or may be suitably prepared as membrane-bound constituents of the microsomes. Such membrane-bound substituents may be introduced using recombinant DNA techniques,
20 as discussed above, to engineer expression of the cytokine in the cell organelle that will ultimately be used to form the microsomes, or alternatively the cytokines may be loaded into the microsome membrane or bound to surface proteins.

A membrane bound cytokine expressed in a microsome preparation may be prepared
25 by transfecting an antigen presenting cell with a construct comprising a cytokine molecule fused to a membrane anchor protein, optionally with an ER-retention signal. For example, a microsome including membrane bound IL-2 molecules can be prepared by constructing a vector comprising nucleic acid encoding a fusion protein comprising the CD2 membrane domain fused to the C-terminus of IL-2 and an ER-retention signal, such as the 16 amino-acid sequence from E15-9K adenovirus protein,
30 where the ER-retention signal is fused to the C-terminus of the CD2 protein. Expression of the vector in the antigen presenting cell leads to accumulation of the

cytokine in the organelles of the cell, i.e. the ER, which enables preparation of microsomes containing membrane-bound cytokine.

5 In addition, it may be convenient to include detection and monitoring of the specific immune responses towards the vaccine, for example by techniques such as ELISA for detection of serum cytokine, e.g. IL-2 and/or IFN γ , or an *in vitro* T cell response assay with peptide loaded microsomes, or a proliferative cell assay.

10 In its simplest form, the present invention provides a composition comprising an isolated microsome of the endoplasmic reticulum of an animal cell, or a membrane fragment thereof, in association with an externally disposed peptide antigen and a protein of the Major Histocompatibility Complex (MHC). Suitably formulated for administration as a vaccine.

15 According to a second aspect of the invention, there is provided a composition according to the first or second aspects of the invention for use in medicine. This aspect of the invention therefore extends to a method of treatment or prophylaxis of a subject suffering from a disease or condition, comprising the step of administering to the subject a vaccine as defined above.

20 According to a third aspect of the invention, there is provided the use of a composition as defined above in the preparation of a vaccine for the prophylaxis or treatment of a disease condition. The disease may be an infection caused by a micro-organism or virus, or it may be a cancer which is characterised by neoplastic cell growth and/or tumour formation. Alternatively, the disease may be an autoimmune condition, where a vaccine may have therapeutic use in inducing tolerance to self-antigens. Uses in accordance with this aspect of the invention also extend to methods of treatment of such disease conditions comprising administering said compositions to a subject in need thereof. Suitably, vaccine compositions of the present invention can be administered by any convenient route such as intramuscular, intravenous, intraperitoneal, oral or by injection in to the cerebrospinal fluid.

25

30

Diseases or conditions that can be treated using a vaccine of the present invention include, but are not limited to melanoma, lung adenocarcinoma, colon cancer, breast cancer or leukemia. Auto-immune disorders include, but are not limited to, Multiple Sclerosis (MS), Systemic Lupus Erythematosus, Type-1 or Insulin-dependent Diabetes, Antiphospholipid Syndrome, Myasthenia Gravis, Myositis, Sjogren's Syndrome and Rheumatoid arthritis. In addition, viral infection, such as HIV infection, herpes virus infection, hepatitis C virus infection, or parasite infections, such as protozoan parasite infection of *Plasmodium*, the causative agent responsible for malaria, for example *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium berghei*, *Plasmodium yoelii* or *Plasmodium knowlesi*, or another parasite such as *Toxoplasma gondii*, or *Trypanosoma brucei*, or *Entamoeba histolytica*, or *Giardia lamblia*, or bacterial infection, such as *E. coli* 0157, *Vibrio cholerae*, etc.

It is also envisaged that the microsomes of the present invention when loaded with peptide antigen may be fused with antigen presenting cells (APC) prior to administration of the combined preparation to the patient. Suitably, the APC are taken from the patient prior to treatment, but may also be taken from an allogenic source. In such cases, where an allogenic source of cells is used, immunosuppressive drugs may also form part of the treatment protocol.

According to a fourth aspect of the invention, there is provided a process for the preparation of a vaccine composition as defined above, the process comprising incubating a population of microsomes and an antigenic peptide in the presence of a nucleoside triphosphate (NTP), followed by further processing to prepare inverted microsomes and formulating the resulting preparation in an physiological diluent and optionally an adjuvant. Reagents such as glucose have ability to preserve the conformation of prepared microsome vaccine and may be included. Suitably, the incubated microsomes will be washed and resuspended in a vaccine solution of an physiological diluent containing an amount of antigenic peptides for preventing the dissociation of the MHC-peptide complex present in the microsome membrane.

The process of loading the microsomes with antigen is carried out in the presence of a nucleoside triphosphate (NTP), such as ATP, GTP, CTP, TTP, or UTP. The antigen loading process may also be carried out in the presence of more than one type of antigenic peptide. In this way a plurality of antigens can be loaded into the
5 microsome.

Inverted microsomes may be prepared by further processing of the microsomes that disrupts the membranes of the microsomes under conditions which allow the membrane to reform and which encourage the formation of "inside-out" microsomes.
10 The use of repeated freeze-thaw steps are therefore suitable in this regard. For example, the microsomes can be suspended in a suitable medium, or buffer, e.g. Phosphate buffered saline (PBS), and then briefly immersed into liquid nitrogen for a suitable period of time, suitably from one to five minutes, preferably for two minutes, and then moved to 37°C, such as in a water-bath, for a suitable period of time,
15 suitably for two to six minutes, preferably for four minutes. The number of repeated steps of freeze-thaw may be from three to five, suitably four repeat steps.

In an alternative embodiment of this aspect of the invention, the process may comprise the loading of inverted microsomes, prepared as described above, with antigen. For loading antigenic peptides into inverted microsomes, the presence of an
20 NTP may not be necessary (although it may be desirable).

According to a fifth aspect of the invention, there is provided a kit of parts comprising a composition as defined above and one or more cytokine and/or adjuvant in sealed
25 containers. Suitably, the kit will comprise instructions for use in a method or use of the invention as defined above.

According to a sixth aspect of the invention, there is provided a kit of parts comprising a composition as defined above and one or more cytokine and/or adjuvant
30 molecules for separate, subsequent or simultaneous administration to a subject.

Preferred features for the second and subsequent aspects of the invention are as for the

first aspect *mutatis mutandis*.

In a particularly preferred embodiment of the invention there is provided a vaccine composition for the prophylaxis or treatment of a disease that can be characterised by the expression of a defined antigen or a peptide sequence which is potentially immunogenic by an infectious agent or which is characterised by the expression of an antigen of a native cell, in which the composition is prepared by:

- (1) obtaining a sample of antigen presenting cells which express MHC proteins;
- (2) homogenising the cells under conditions such that a preparation of microsomes is isolated;
- (3) preparation of antigenic peptides, for example by means of recombinant DNA technology, or from isolation from a natural tissue source, or source of infectious agent, or in most cases synthesised antigenic peptides.
- (4) incubation of antigenic peptides and microsomes in the presence of an NTP to load the microsomes with antigenic peptides;
- (5) further processing of microsomes loaded in step (4) to prepare a population of inverted microsomes
- (6) formulation of loaded inverted microsomes a vaccine in a physiological diluent and/or adjuvant as appropriate

As described above, the microsomes may also be prepared from an isolated population of cells or a cell line. The cells or cell line may have been transfected with a nucleic acid construct to express a protein of choice prior to microsome preparation.

According to the above protocol, the microsomes are loaded with antigenic peptide and then subsequently processed to prepare inverted microsomes. However, in an alternative embodiment, the inverted microsomes may be prepared first and then

loaded with antigen, in which case the presence on an NTP in step (4) may not be required.

5 As discussed above, the vaccine compositions of the invention preferably comprise inverted microsomes, more preferably a homogenous population of inverted microsomes. However, non-inverted microsomes may also be present in the population of inverted microsomes.

10 The cells may be MHC negative so as to permit transfection of the cell line with appropriate nucleic acid encoding the MHC class molecule of choice for the vaccine. Preparation of antigen may also include synthesis of antigenic peptides by means of chemical means.

15 The loading of non-inverted microsomes with antigenic peptide takes place in the presence of an NTP. The loading of inverted microsomes does not appear to require the presence of an NTP, although it may be preferred.

20 The invention will now be further described by way of reference to the following Examples and Figures which are provided for the purposes of illustration only and are not to be construed as being limiting on the invention. Reference is made to a number of Figures in which:

25 FIGURE 1 shows crosslinking of H2-Kb molecules by crosslinker-modified OVA peptide in the microsomes of RAW309Cr.1 cells. The ¹²⁵I-labeled ANB-NOS-OVA peptide was mixed with the microsomes of RAW309Cr.1 cells in the presence or absence of ATP-regenerating system or of native OVA-peptide at a ten-fold molar excess. The crosslinked H-2Kb was indicated.

30 FIGURE 2 shows concentration of OVA-peptide receptive H-2Kb in the microsomes of RAW309Cr.1 cells. For semi-quantitation of OVA-peptide receptive H-2Kb, 10 nMs of labelled peptide was incubated with the

microsomes or RAW309Cr.1 cells, respectively, under the UV irradiation. The H-2 molecules were precipitated by R218 antiserum and crosslinked Kb molecules were quantitated by phospho-imaging.

5 FIGURE 3 shows detection of H-2 molecules in the microsomes or on the surface of RAW309Cr.1 cells. 30 µg proteins from NP40 lysates of RAW309Cr.1 microsomes or RAW309Cr.1 cells were separated on 10% SDS-PAGE. The lysates were diluted at the titration indicated and separated on the SDS-PAGE. Immunoblotting of H-2 molecules was detected by R218
10 anti-H-2 antiserum.

FIGURE 4 shows detection of B7.1, B7.2 and ICAM-1 in the microsomes of RAW309Cr.1 cells. 30 µg proteins from NP40 lysates of RAW309Cr.1
15 microsomes or RAW309Cr.1 cells were separated on 10% SDS-PAGE. Immunoblotting of B7.1, B7.2 and ICAM-1 was detected by specific antibodies.

FIGURE 5 shows stimulation of B3Z T cells by OVA-peptide edited
20 microsomes. Microsomes from 2×10^5 RAW309Cr.1 cells were used to load OVA or Ld-specific peptide as described in Material and Methods. 2×10^5 RAW309Cr.1 cells were pulsed with OVA peptide (see Material and Methods). After washing, peptide-pulsed 2×10^5 RAW309Cr.1 cells, OVA-loaded microsomes, Ld-peptide loaded microsomes, and the microsomes without peptide were co-cultured with 10^5 B3Z cells for over night. A) After
25 washing with PBS, LacZ activity in B3Z cells was assayed by total cellular lysates with the LacZ substrate ONPG. The absorbance (415 nm) was read after incubation for four hours at 37°C. B3Z cells cultured with 100 nM OVA peptide and normal medium were used as positive and negative control for the B3Z stimulation. B) The supernatants of these cultures were submitted for
30 measuring IL-2 production by ELISA. The experiment was repeated four times with similar results. Error bars indicate the SEM of triplicate cultures.

FIGURE 6 shows stimulation of B3Z T cells by the microsomes of 2×10^5 RAW309Cr.1 cells pre-loaded with different concentrations of OVA-peptides. Microsomes loaded with OVA-peptide at different concentration indicated were co-cultured with B3Z cells overnight before the assay of LacZ activity.

5

FIGURE 7 shows OVA-peptide edited microsomes stimulates specific T cell responses *in vivo*. C57BL/6 (H-2b) mice were primed i.s. by OVA-edited microsomes or Ld-peptide loaded microsomes or OVA peptide or OVA-pulsed RAW309Cr.1 cells and challenged by same stimulus after seven days. Six days after the challenge, enriched T cells were isolated from spleens and cultured at 10^5 cells/well with stimulus indicated. The RAW309Cr.1 cells were irradiated before co-culture with T cells. After three days, supernatants were harvested for cytokine ELISA (b) and cultures pulsed with [3 H]thymidine (a). The results are representative of groups of at least three mice per treatment group and the experiment was repeated four times with similar results. Error bars indicate the SEM of triplicate cultures. Similar set of experiments performed in Balb/c (H-2d) mice served as negative control.

10

15

20

FIGURE 8 shows activation of TCR induced MAK kinases. 10^7 T cells from OVA-microsomes immunised C57BL/6 were stimulated with OVA-pulsed RAW309Cr.1, OVA-microsomes and anti-CD3/CD28, respectively. Activation of ERK and JNK was detected by anti-p-ERK and anti-p-JNK antibodies. Similar levels of ERK and JNK detected by anti-ERK and anti-JNK served as loading control.

25

FIGURE 9 shows OVA-receptive H-2Kb detected in microsomes, but not on the surface of RAW309Cr.1 cells. The 125 I-labeled ANB-NOS-OVA peptide 10 nM was mixed without or with native OVA peptide at concentrations indicated. The mixed peptides were incubated with microsomes equivalent to 10^7 RAW309Cr.1 cells or with 10^7 RAW309Cr.1 cells. The crosslinked H2-Kb molecules were indicated.

30

FIGURE 10 shows the results of a study in which influenza viral peptide edited Kb-microsomes induced T-cell responses *in vivo* (five mice in each group).

5 FIGURE 11 shows electronmicrograph pictures of DC and prepared microsomes: (A) magnification x12000 and (B) magnification x40000 are prepared microsomes from RAW cells; (C) magnification x40000 are microsomes inverted by repeated freeze-thaw and loaded with peptides, showing open or inverted microsomes. The loaded peptides can not be seen in
10 the picture.

FIGURE 12 shows the results of a study carried out on MAGE-A2 specific T-cells from A2 melanoma patients.

15 FIGURE 13 shows the amino acid sequences for the MHC class I antigens A2 alpha chain precursor and B7 alpha chain precursor (Accession nos. P01892 and P01889, respectively).

20 FIGURE 14 shows the amino acid sequences for the MHC class II antigens DRB3-1 beta-chain precursor and HLA-DQ alpha 1 (DQw4 specificity) precursor – human (Accession nos. P79483 and A37044, respectively).

Material and Methods

Cell lines and animals

25 B3Z is a CD8 T cell hybridoma that expresses LacZ in response to activation of T cell receptors specific for the SIINFEKL peptide presented by H-2Kb MHC class I molecules. RAW309Cr.1, a Kd/Kb murine macrophage cell line, used as APCs, was obtained from ATCC (ATCC TIB-69). All cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Female C57BL/6 mice H-2b and Balb/c
30 mice H-2d were obtained at 6 weeks of age. All procedures with animals were carried out in accordance with approved protocols.

Antibodies, peptides, and peptide modification

All peptides were synthesised in a peptide synthesiser (model 431A, Applied Biosystems, Foster City, CA), using conventional F-moc chemistry, and were subsequently purified by HPLC. The purified peptides were dissolved in PBS.

5

Peptide OVA 257-264 (SIINFEKL) was modified by substitution of third residue isoleucine to tyrosine in order for iodination and by covalently coupling a phenylazide with a nitro group on the ϵ -amino group of lysine at position seven. This nitro group can be photoactivated. The crosslinker modification was performed by mixing 0.5 mg of ANB-NOS (N-5-azido-2-nitrobenzoyloxysuccinimide) in 200 μ l DMSO with 100 μ g peptide in 100 μ l PBS and 50 μ l CPAS (3-[cyclohexylamino]-1-propanesulfonic acid) (0.5 M, pH 10). The reaction was allowed to proceed for 30 min on ice. To remove the excess ANB-NOS and ions, the mixture was purified by gel filtration on Sephadex G-10 and subsequently by HPLC. An aliquot (1 μ g) of the peptide was labelled by chloramines-T-catalyzed iodination (125 I). The modification and labelling experiments were performed in the dark.

10

15

Antisera, immunoprecipitation, and SDS-PAGE

Rabbit antiserum to H2 (R218) was kindly provided by Dr. Sune Kvist, Karolinska Institute. Monoclonal antibody specific to confirmed H2 (Y3) was kindly provided by Tim Elliot, Cambridge University. Antisera to JNK, ERK, p-ERK and p-JNK were obtained from (Santa Cruz Biotechnology). Immunoprecipitation, immunoblotting and SDS-PAGE were performed as described in Li *et al* (*J Biol Chem.* **274** (13), 8649-54 (1999)). Protein-A-Sepharose was obtained from Pharmacia (Uppsala, Sweden).

20

25

Example 1: Preparation of microsomes and peptide binding assay

Microsomes from RAW309Cr.1, a Kd/Kb murine macrophage cell line were prepared and purified according to the procedure of Saraste *et al* (*Proc. Natl. Acad. Sci. U. S. A.* **83**, 6425-6429 (1986)). The immunogenetics of class I is Kb in RAW cells and Balb/c mice.

30

Preparation of microsomes from B cells based on a modification of Saraste *et al* (*Proc. Natl. Acad. Sci. U. S. A.* 83, 6425-6429 (1986)) and Knipe *et al* (*J. Virol.* 21, 1128-1139 (1977)) for fractionation of microsomal membranes was used. All steps were performed at 0-4 °C).

5

- 3×10^9 cells are collected and washed once with cold PBS.
- Resuspend the cells in 20 ml STKMM-buffer with 10 μ l of PMSF (100mM).
- Spin at 1500rpm for 5 min at 4°C.
- Resuspend in 10 ml H₂O (with 5 μ l PMSF).

10

- Homogenise in 40 ml Dounce, 20 strokes.
- Add 30 ml STKMM and mixing well.
- Pour over in JA-18 tubes.
- Centrifuge at 7500rpm for 10 min at 4°C.
- Carefully collect supernatant to the new tubes.

15

- Centrifuge at 15500rpm for 54 min at 4°C.
- Carefully wash the pellet with 10 ml STKMM buffer, then resuspend the pellet in 1 ml RM buffer with a pipette and homogenise in 15ml douncer. The rough microsomes will be diluted at a concentration of $A_{OD280} = 60$.

20

- Total microsomes (described above) were layered on top of 5 ml of 0.33 M sucrose containing 5 mM benzamidine, layered in turn on top of a sucrose cushion consisting of 1 ml of 2 M sucrose/5 mM benzamidine.

25

- Centrifugation in an SW41 rotor for 60 min at $110,000 \times g$ yielded a total microsome band on top of the cushion. The total microsome band was carefully collected. Then, 2 M sucrose/5 mM benzamidine was slowly added to the microsomes to give a final concentration of 45% (w/v) sucrose.

30

- Microsomes were subfractionated by flotation using a modification of the method described in Paulsson *et al* (*J Biol Chem.* 277 (21), 18266-71 (2002)). 100 μ l of the total microsomes in 3 ml of 45% (w/v) sucrose was placed at the bottom of an SW41 ultracentrifuge tube and overlaid with the following sucrose solutions: 1 ml of 30% and 1.9 ml each of 27.5%, 25%, 22.5%, and 20.0% (all solutions contained 5 mM benzamidine).

- After centrifugation at 4 °C for 10 h at 37,000 rpm (to reach isopycnic conditions), 25 fractions of 300 µl each were collected by upward displacement.
- The ER fractions will be determined by western blotting with anti-p58 antibody. (p58 is a ER protein).
- The poured ER fractions will be used in peptide-loading and immunisation experiments.

The cross-link mixture contained 50 or 100 nM (¹²⁵I)ANB-NOS-peptide and 10 µl of microsomes (60 A₂₈₀/ml) in a total volume of 100 µl RM buffer (250 mM sucrose, 50 mM TEA-HCl, 50 mM KOAc, 2 mM MgOAc₂, and 1mM DTT). After mixing, the samples were immediately irradiated at 366 nm for 5 min at room temperature. The membranes were then recovered by centrifugation through a 0.5-M sucrose cushion in RM buffer. The membranes were washed once with cold RM buffer. The washing membranes were lysed for immunoprecipitation or for immune blotting. The crosslinking reaction with ATP contained an ATP regeneration system, described in Li *et al* (*J Biol Chem.* 274 (13), 8649-54 (1999)). The crosslinking of surface Kb molecules on RAW309Cr.1 cells was performed as mixing 100 nM (¹²⁵I)ANB-NOS-peptide with 10⁷ cells, equivalent to amount of cells used for making 10 µl of microsomes in a total volume of 100 µl RM buffer. After mixing, the samples were immediately irradiated for 5 min at room temperature. The excess peptides were removed by washing with RM buffer. The cells were lysed for immunoprecipitation with Y3 antibody.

The detection of surface MHC class I was performed by incubating RAW309Cr.1 cells with Y3 antibody at 4°C for 15 min. After washing, the cells were lysed in 1% NP40 lysis buffer and the cleared lysates were precipitated with protein-A beads. The precipitated MHC class I were detected by immunoblotting with R218 antiserum.

The peptide-editing for stimulation of T cells was performed by mixing microsomes with native peptides with ATP regeneration system for 10 min at room temperature.

The excess of peptides was removed after centrifugation through sucrose cushion in RM buffer. The loaded microsomes were repeatedly freeze/thaw alternately in liquid nitrogen and then in a water bath at 37°C, for 10 times. The processed microsomes were resuspended in PBS at concentration of (6 A₂₈₀/ml) and kept at -80°C until use.

- 5 The peptide-pulsed RAW309Cr.1 cells was prepared as mixing peptides 100 nM with 10⁷ cells in 1 ml medium over night or in 1 ml PBS for four hours at 37°C. The pulsed cells were either washed with PBS before mixing with B3Z T cells or add the mixture directly to the B3Z.

10 **Example 2: Activation of B3Z T cell hybridoma**

- The prepared stimuli including peptide-edited microsomes, peptide-pulsed RAW309Cr.1 cells, OVA peptide, were added to culture of 10⁵ B3Z cells in a total of 200 µl. Addition of PBS and anti-CD3/CD28 coated beads served as negative or positive control, respectively. After over night incubation, the activation of B3Z was represented by LacZ activity using o-nitrophenyl b-D-galactopyranoside (Sigma) substrate. The linear range of OVA-response was determined by the addition of serial dilutions of SIINFEKL to the medium due to that the B3Z cells themselves express Kb and present SIINFEKL.
- 15

20 **Example 3: Detection of peptide-receptive MHC class I molecules in the microsome, but not on the surface of APCs.**

- An *in vitro* peptide transport and loading assay by using crosslinker modified peptides and isolated microsomes of the ER from RAW309Cr.1 has been reported (Li *et al J Biol Chem.* 274 (13), 8649-54 (1999)). The assay allows the examination of both the peptide translocation across the membrane of the ER in the presence of ATP and subsequently the peptide loading on MHC class I molecules (Wang *et al J Immunol.* 157 (1), 213-20 (1996)).
- 25

- To detect the peptide-receptive MHC class I molecules in the microsomal membranes, a crosslinker (ANB-NOS) was conjugated to the ε-amino group of the lysine residue of an H2-Kb-binding ovalbumin (OVA) peptide (residues 257-264, SIIFEKL) and substituted the isoleucine at position 3 with tyrosine to allow for iodination. These
- 30

modifications allowed photo-cross-linking of the OVA peptide to H2-Kb molecules during the assembly. For a quantitative comparison of peptide-receptive H2-Kb in microsomes and on cell surface of living RAW309Cr.1, the modified OVA peptide was labelled by ^{125}I and incubated with microsomes of RAW309Cr.1 and living
5 RAW309Cr.1 cells under UV irradiation. Peptide-bound H2-Kb molecules were subsequently analysed by immunoprecipitation with an anti-H2 antibody Y3. In the absence of ATP, only a few Kb molecules were assembled with OVA peptides, while a significant amount of Kb molecules were cross-linked with OVA peptide in the presence of ATP (Fig. 1). This result confirms that a substantial amount of peptide
10 receptive class I molecules exist in the ER.

A semi-quantitative analysis of OVA-crosslinked Kb in microsomes and on the surface of RAW309Cr.1 showed that in contrast to the high levels of peptide receptive Kb molecules in the microsomes, the OVA-bound Kb molecules on the surface of
15 APCs was under the radio-chemical detective level (Fig. 2), suggesting again that peptide-receptive MHC class I molecules are mainly in the ER, but not on the surface of APCs. In a competing experiment, it has been shown that the binding of this modified OVA peptide to Kb is specific. In order to examine the affinity of the modified OVA-peptide, the labelled OVA peptide was competed by its native form at
20 different concentrations. The native OVA peptide competed 50% of the report peptide at the concentration of report peptides and completely abolished binding at concentration of ten times of the report peptides (Fig. 2). Moreover, a Ld specific peptide could not compete the OVA binding. This shows that the binding affinity of modified OVA-peptide is Kb specific and similar to its native form. To quantitate the
25 amount of peptides bound to Kb molecules in microsomes derived from 10^6 RAW309Cr.1, the labelled peptides were incubated with microsomes in the presence of ATP. After crosslinking, MHC class I were precipitated and dpm of peptide-bound Kb was measured and converted to the concentration of peptides. Results showed that about 500 to 1000 peptides were bound to Kb molecules in the microsomes of one
30 cells. In addition, the amount of total MHC class I molecules in the ER are more than that on the surface of RAW309Cr.1 cells (Fig. 3). Thus, microsomes from APCs could be able to deliver sufficient peptide-MHC class I complexes to T cells.

Example 4: B7 and ICAM1 are presented in the microsomes of APCs.

A full T cell response requires signals from both antigen-MHC complex and co-stimulatory molecules such as B7 (Acuto *et al*, *Immunol Rev.* **192**, 21-31 (2003)).
5 Like all the membrane proteins, co-stimulatory molecules are synthesised in the ER and subsequently expressed on the surface of APCs. To quantitate the amount of co-stimulatory molecules of B7 and ICAM-1 in the isolated microsomes, the microsomes equivalent to 5×10^6 RAW309Cr.1 were lysed and the clear lysates were analysed by western blotting with anti-sera specific to these molecules, respectively. In
10 comparison, a total cell lysates of 5×10^6 RAW309Cr.1 were also blotted with same antibody. The intensity of B7.1, B7.2 and ICAM-1 bands was quantitated by density analysis. Both B7 and ICAM-1 were readily detected in the microsomal samples (Fig. 4). The amount detected in microsomes was about half of the total cellular lysates. The presence of sufficient amount of co-stimulatory molecules in peptide-edited
15 microsomes could mimic the functional surface of APCs for providing both antigen-MHC and co-stimulatory signals to T cells.

Example 5: Microsomes loaded with Kb-specific OVA peptides stimulate T cells in vitro

20 To investigate the ability for peptide-loaded microsomes to induce specific T cell response, the native OVA peptide-edited microsomes were processed for inside-out by repeated freeze-thaw method (materials and methods). The processed microsomes and OVA-peptide pulsed RAW309Cr.1 were used to stimulate B3Z T cell hybridoma which recognises Kb-SIINFEKL complex (Fremont *et al Proc Natl Acad Sci U S A.*
25 **92** (7), 2479-83 (1995); Shastri N, & Gonzalez F., *J Immunol.* **150** (7), 2724-36 (1993)). After washing off the excessive peptide, OVA edited Microsome stimulated B3Z T cells by inducing IL-2 production and the expression of IL-2-promoter driven LacZ (Fig. 5). The specificity of OVA-Kb induced B3Z responses was supported by the unresponsiveness of B3Z cells to the microsomes without the peptide or loaded
30 with Ld specific peptide (Fig. 5). Moreover, the levels of responses of B3Z to OVA-edited microsomes was correlated with the amount of OVA-peptides (Fig. 6). OVA-

pulsed RAW309Cr.1 could induce the B3Z response in the presence of excessive peptides (Schott *et al Proc Natl Acad Sci U S A.* 99 (21), 13735-40 (2002)).

5 However, if excess peptides were removed by washing, the OVA-pulsed-RAW309Cr.1 could no longer induce B3Z response. Given that OVA itself could induce IL-2 production by B3Z cells (Fig. 5), suggests that not RAW309Cr.1, but OVA itself is the stimuli for B3Z. The ability of SIINFEKL to induce Kb restricted T cell responses *in vitro* has been reported recently, suggesting that CTL could present peptides to each other (Schott *et al Proc Natl Acad Sci U S A.* 99 (21), 13735-40
10 (2002)). However, the induction CTL response *in vitro* by peptide-edited microsomes, but not by peptide-pulsed RAW309Cr.1 is consistent with the peptide-binding results (Fig. 2) and indicate that peptide-edited microsomes could mimic APCs to efficiently present antigenic peptides to TCR and stimulate full responses of CTLs.

15 **Example 6: Microsomes loaded with Kb-specific OVA peptides induces OVA-peptide responses *in vivo***

To further examine the ability of OVA-edited microsomes to induce immune responses *in vivo*, OVA-edited microsomes from RAW309Cr.1 cells, microsomes loaded with Ld-specific peptide, soluble OVA peptides, RAW309Cr.1 pulsed with
20 OVA peptides and PBS were used to induce immune response *in vivo*. Five groups of C57BL/6 or Balb/c mice, each group consisting of five mice, were injected twice subcutaneously with above stimuli, respectively. The interval between injections was one week. Six days after second injection, T cells were isolated from spleens and cross-stimulated *in vitro* with the five original stimuli, respectively. In addition, anti-
25 CD3/CD28 coated beads were used as positive control. PBS stimulated T cells did not respond to any stimulation, while anti-CD3/CD28 induced proliferative responses in all the groups. OVA-peptide pulsed RAW309Cr.1 and microsomes loaded with Ld-specific peptide did not induce T cells responses (Fig. 7). In contrast, T cells from C57BL/6 groups of OVA-edited microsomes and OVA peptide responded to OVA-
30 edited microsomes *in vitro*, but not to the OVA-pulsed RAW309Cr.1 or the microsomes loaded with Ld-peptides (Fig. 7). Compelling results from IL-2 production (Fig.7) again support that OVA-edited microsomes could induce specific

T cells responses *in vivo* (Fig. 7). Balb/c has H-2d, therefore, there was not OVA response induced.

Example 7: TCR signalling pathways are activated by OV-microsomes

- 5 In order to analyse the TCR signalling in response to OVA-microsome stimulation, T cells isolated from C57BL/6 mice immunised by OVA-microsomes were used to induce TCR signalling *in vitro*. The activation of ERK and JNK was detected in the T cells stimulated with either anti-CD3/CD28 or with OVA-edited microsomes, but not with OVA-pulsed RAW309Cr.1 (Fig. 8). Thus, the biochemical evidence indicates a
- 10 specific TCR signalling in response to OVA-Kb on microsomes and further supports that microsomes edited with antigenic peptides could induce specific immune responses *in vivo*.

Example 8: Influenza viral peptide loaded microsomes

- 15 The Kb specific peptide (ASNENMETM) from mouse influenza virus was loaded into microsomes from Kb specific RAW cells. The loaded microsomes were used to immunize C57BL/6 mice. In separate groups, the PBS or peptide were used as controls. After two antigen administrations over a seven day period, T-cells were isolated from spleens and cross-cultured with PBS or peptide, peptide, or peptide-
- 20 loaded microsomes for three days. T-cell proliferation was measured on day 4 after immunization. Results are shown in Figure 10.

Example 9: Effect on melanoma cells

- 25 T-cells isolated from three A2 melanoma patients (P1, P2, P3), respectively, were stimulated with autologous tumor cells purified from surgical biopsies at one to one and with r-human IL-2 (10 U/mL) for four times with a 5 day interval between each administration. The specific anti-tumor responses were tested in comparison to the tumor cell line K259 by cytotoxic assay. The MAGE peptide was loaded to microsomes isolated from 221-A2 human B-cell line, in which the MHC locus is
- 30 deleted, and subsequently transfected with HLA-A2. The T-cell lines from melanoma patients were cultured with peptide, microsomes, or peptide-loaded microsomes in

normal medium for three days. The proliferation was measured at day 3. The results are shown in Figure 12.

Discussion

- 5 These results demonstrate that the microsomes derived from the ER can be used to process edited antigenic peptides on MHC class I molecules and that the processed microsomes can reconstitute the functional surface of APCs to induce CTL responses. Thus, the antigenic peptide delivered by microsomal MHC class I in association with co-stimulatory molecules is a novel form of peptide vaccine.

10

References to Tables 1 to 7

1. Aarnoudse *et al*, *Int J Cancer* **82**: 442 (1999)
2. Anichini *et al*, *J Exp Med* **177**: 989 (1993)
3. Bakker *et al*, *Int J Cancer* **62**: 97 (1995)
- 15 4. Baurain *et al*, *J Immunol* **164**: 6057 (2000)
5. Bocchia *et al*, *Blood* **87**: 3587 (1996)
6. Boel *et al*, *Immunity* **2**: 167 (1995)
7. Bohm *et al*, *Int J Cancer* **75**: 688 (1998)
8. Boon *et al*, *J Exp Med* **183**: 725 (1996)
- 20 9. Brandle *et al*, *J Exp Med* **183**: 2501 (1996)
10. Brichard *et al*, *Eur J Immunol* **26**: 224 (1996)
11. Brossart *et al*, *Blood* **93**: 4309 (1999)
12. Butterfield *et al*, *Cancer Res* **59**: 3134 (1999)
13. Buzyn *et al*, *Eur J Immunol* **27**: 2066 (1997)
- 25 14. Castelli *et al*, *J Exp Med* **181**: 363 (1995)
15. Castelli *et al*, *J Immunol* **162**: 1739 (1999)
16. Chaux *et al*, *J Immunol* **163**: 2928 (1999a)
17. Chaux *et al*, *J Exp Med* **189**: 767 (1999b)
18. Chen *et al*, *Proc Natl Acad Sci USA* **94**: 1914 (1997)
- 30 19. Chiari *et al*, *Cancer Res* **59**: 5785 (1999)
20. Corman *et al*, *Clin Exp Immunol* **114**: 166 (1998)
21. Correale *et al*, *J Natl Cancer Inst* **89**: 293 (1997)

22. Coulie *et al*, *J Exp Med* **180**: 35 (1994)
23. Coulie *et al*, *Proc Natl Acad Sci USA* **92**: 7976 (1995)
24. Cox *et al*, *Science* **264**: 716 (1994)
25. Dabovic *et al*, *Mamm Genome* **6**: 571 (1995)
- 5 26. De Backer *et al*, *Cancer Res* **59**: 3157 (1999)
27. De Plaen *et al*, *Immunogenetics* **40**: 360 (1994)
28. Dermime *et al*, *Clin Cancer Res* **2**: 593 (1996)
29. De Smet *et al*, *Immunogenetics* **19**: 121 (1994)
30. De Smet *et al*, *Mol Cell Biol* **19**: 7327 (1994)
- 10 31. Domenech *et al*, *J Immunol* **155**: 4766 (1995)
32. Dudley *et al*, *J Exp Med* **184**: 441 (1996)
33. Duffour *et al*, *Eur J Immunol* **29**: 3329 (1999)
34. Fisk *et al*, *J Exp Med* **181**: 2109 (1995)
35. Fleischhauer *et al*, *Int J Cancer* **68**: 622 (1996)
- 15 36. Fleischhauer *et al*, *Cancer Res* **58**: 2969 (1998)
37. Fujie *et al*, *Int J Cancer* **80**: 169 (1999)
38. Gambacorti-Passerini *et al*, *Blood* **81**: 1369 (1993)
39. Gaudin *et al*, *J Immunol* **162**: 1730 (1999)
40. Gaugler *et al*, *J Exp Med* **179**: 921 (1994)
- 20 41. Gaugler *et al*, *Immunogenetics* **44**: 323 (1996)
42. Gorni *et al*, *J Immunol* **163**: 4994 (1999)
43. Greco *et al*, *Leukemia* **10**: 693 (1996)
44. Gueguen *et al*, *J Immunol* **160**: 6188 (1998)
45. Guilloux *et al*, *J Exp Med* **183**: 1173 (1996)
- 25 46. Gure *et al*, *Int J Cancer* **85**: 726 (2000)
47. Heidecker *et al*, *J Immunol* **164**: 6041 (2000)
48. Herman *et al*, *Immunogenetics* **43**: 377 (1996)
49. Hiltbold *et al*, *Cancer Res* **58**: 5066 (1998)
50. Hogan *et al*, *Cancer Res* **58**: 5144 (1998)
- 30 51. Hohn *et al*, *J Immunol* **163**: 5715 (1999)
52. Huang *et al*, *J Immunol* **162**: 6849 (1999)
53. Ikeda *et al*, *Immunity* **6**: 199 (1997)

54. Jager *et al*, *J Exp Med* **187**: 265 (1998)
55. Jager *et al*, *J Exp Med* **191**: 625 (2000)
56. Jassim *et al*, *Eur J Immunol* **19**: 1215 (1989)
57. Kang *et al*, *J Immunol* **155**: 1343 (1995)
5 58. Kawakami *et al*, *Proc Natl Acad Sci USA* **91**: 3515 (1994a)
59. Kawakami *et al*, *Proc Natl Acad Sci USA* **91**: 6458 (1994b)
60. Kawakami *et al*, *J Exp Med* **180**: 347 (1994c)
61. Kawakami *et al*, *J Immunol* **154**: 3961 (1995)
62. Kawakami *et al*, *J Immunol* **161**: 6985 (1998)
10 63. Kawakami *et al*, *Immunol Res* **16**: 313 (1997)
64. Kawano *et al*, *Cancer Res* **60**: 3550 (2000)
65. Kawashima *et al*, *Int J Cancer* **78**: 518 (1998)
66. Kawashima *et al*, *Cancer Res* **59**: 431 (1999)
67. Kikuchi *et al*, *Int J Cancer* **81**: 459 (1999)
15 68. Kittlesen *et al*, *J Immunol* **160**: 2099 (1998)
69. Kobayashi *et al*, *Cancer Res* **58**: 296 (1998a)
70. Kobayashi *et al*, *Immunogenetics* **47**: 398 (1998b)
71. Kono *et al*, *Int J Cancer* **78**: 202 (1998)
72. Lethe *et al*, *Int J Cancer* **76**: 903 (1998)
20 73. Li *et al*, *Cancer Immunol Immunother* **47**: 32 (1998)
74. Lucas *et al*, *Cancer Res* **59**: 4100 (1999)
75. Lucas *et al*, *Int J Cancer* **87**: 55 (2000)
76. Lupetti *et al*, *J Exp Med* **188**: 1005 (1998)
77. Lurquin *et al*, *Genomics* **46**: 397 (1997)
25 78. Mandruzzato *et al*, *J Exp Med* **186**: 785 (1997)
79. Manici *et al*, *J Exp Med* **189**: 871 (1999)
80. Martelange *et al*, *Cancer Res* **60**: 3848 (2000)
81. Minev *et al*, *Proc Natl Acad Sci USA* **97**: 4796 (2000)
82. Moreau-Aubry *et al*, *J Exp Med* **191**: 1617 (2000)
30 83. Morel *et al*, *Immunity* **12**: 107 (2000)
84. Morioka *et al*, *Mol Immunol* **32**: 573 (1995)
85. Nakao *et al*, *J Immunol* **164**: 2565 (2000)

86. Noppen *et al*, *Int J Cancer* **87**: 241 (2000)
87. Norbury *et al*, *Br J Haematol* **109**: 616 (2000)
88. Ohminami *et al*, *Blood* **93**: 925 (1999)
89. Oiso *et al*, *Int J Cancer* **81**: 387 (1999)
- 5 90. Oka *et al*, *Immunogenetics* **51**: 99 (2000)
91. Panelli *et al*, *J Immunol* **164**: 4382 (2000)
92. Parkhurst *et al*, *Cancer Res* **58**: 4895 (1998)
93. Pawelec *et al*, *Blood* **88**: 2118 (1996)
94. Peiper *et al*, *Eur J Immunol* **27**: 1115 (1997)
- 10 95. Peoples *et al*, *Proc Natl Acad Sci USA* **92**: 432 (1995)
96. Pieper *et al*, *J Exp Med* **189**: 757 (1999)
97. Robbins *et al*, *J Immunol* **154**: 5944 (1995)
98. Robbins *et al*, *J Exp Med* **183**: 1185 (1996)
99. Robbins *et al*, *J Immunol* **159**: 303 (1997)
- 15 100. Robbins *et al*, *Harwood Acad Publ, London*, in press (2000)
101. Rongcun *et al*, *J Immunol* **163**: 1037 (1999)
102. Ronsin *et al*, *J Immunol* **163**: 483 (1999)
103. Russo *et al*, *Proc Natl Acad Sci USA* **97**: 2185 (2000)
104. Salazar-Onfray *et al*, *Cancer Res* **57**: 4348 (1997)
- 20 105. Scanlan *et al*, *Cancer Lett* **150**: 155 (2000)
106. Schneider *et al*, *Int J Cancer* **75**: 451 (1998)
107. Shichijo *et al*, *J Exp Med* **187**: 277 (1998)
108. Skipper *et al*, *J Immunol* **157**: 5027 (1996)
109. Suzuki *et al*, *J Immunol* **163**: 2783 (1999)
- 25 110. Tahara *et al*, *Clin Cancer Res* **5**: 2236 (1999)
111. Tanaka *et al*, *Cancer Res* **57**: 4465 (1997)
112. Tanaka *et al*, *Br J Haematol* **109**: 435 (2000)
113. Tanzarella *et al*, *Cancer Res* **59**: 2668 (1999)
114. ten Bosch *et al*, *Leukemia* **9**: 1344 (1995)
- 30 115. ten Bosch *et al*, *Blood* **88**: 3522 (1996)
116. ten Bosch *et al*, *Blood* **94**: 1038 (1999)
117. Topalian *et al*, *Proc Natl Acad Sci USA* **91**: 9461 (1994)

118. Topalian *et al*, *J Exp Med* **183**: 1965 (1996)
119. Traversari *et al*, *J Exp Med* **176**: 1453 (1992)
120. Tsai *et al*, *J Immunol* **158**: 1796 (1997)
121. Tsang *et al*, *J Natl Cancer Inst* **87**: 982 (1995)
- 5 122. van Baren *et al*, *Br J Haematol* **102**: 1376 (1998)
123. Van den Eynde *et al*, *J Exp Med* **182**: 689 (1995)
124. Van den Eynde *et al*, *J Exp Med* **190**: 1793 (1999)
125. van der Bruggen *et al*, *Science* **254**: 1643 (1991)
126. van der Bruggen *et al*, *Eur J Immunol* **24**: 3038 (1994a)
- 10 127. van der Bruggen *et al*, *Eur J Immunol* **24**: 2134 (1994b)
128. Visseren *et al*, *Int J Cancer* **73**: 125 (1997)
129. Vissers *et al*, *Cancer Res* **59**: 5554 (1999)
130. Vonderheide *et al*, *Immunity* **10**: 673 (1999)
131. Wang *et al*, *J Exp Med* **184**: 2207 (1996a)
- 15 132. Wang *et al*, *J Exp Med* **183**: 1131 (1996b)
133. Wang *et al*, *J Immunol* **160**: 890 (1998a)
134. Wang *et al*, *J Immunol* **161**: 3598 (1998b)
135. Wang *et al*, *Science* **284**: 1351 (1999a)
136. Wang *et al*, *J Exp Med* **189**: 1659 (1999b)
- 20 137. Wolfel *et al*, *Eur J Immunol* **24**: 759 (1994)
138. Wolfel *et al*, *Science* **269**: 1281 (1995)
139. Yang *et al*, *Cancer Res* **59**: 4056 (1999)
140. Yasukawa *et al*, *Blood* **92**: 3355 (1998)
141. Yotnda *et al*, *J Clin Invest* **101**: 2290 (1998a)
- 25 142. Yotnda *et al*, *J Clin Invest* **102**: 455 (1998b)
143. Yun *et al*, *Tissue Antigens* **54**: 153 (1999)
144. Zarour *et al*, *Proc Natl Acad Sci USA* **97**: 400 (2000)
145. Zeng *et al*, *J Immunol* **165**: 1153 (2000)
146. Zorn *et al*, *Eur J Immunol* **29**: 592 (1999a)
- 30 147. Zorn *et al*, *Eur J Immunol* **29**: 602 (1999b)

Table 1 Class I HLA-restricted cancer/testis antigens. All these antigens were found to be expressed by normal spermatocytes and/or spermatogonia of testis.

Occasionally *MAGE-3*, *MAGE-4* and the *GAGE* genes were found to be expressed also in placenta [26, 24]. The NY-ESO-1 antigen was found to be expressed in normal ovary cells [18].

<i>Gene</i>	<i>HLA allele</i>	<i>Peptide epitope</i>	<i>Author [Ref]</i>	<i>Tissue distribution among tumors^a</i>
<i>MAGE-A1</i>	A1	EADPTGHSY	Traversari et al., 1992 [119]	Melanoma, breast carcinoma, SCLC [27, 29, 125] -
<i>MAGE-A1</i>	A3	SLFRAVTK	Chaux et al., 1999a [16]	sarcoma, NSCLC [27, 29] - thyroid medullary carcinoma
<i>MAGE-A1</i>	A24	NYKHCFPEI	Fujie et al., 1999 [37]	[125] - colon carcinoma [27] - laryngeal tumors [29]
<i>MAGE-A1</i>	A28	EVYDGREHSA	Chaux et al., 1999a [16]	
<i>MAGE-A1, -A2</i>	B37	REPVTKAEML	Tanzarella et al., 1999 [113]	Melanoma, colon and breast carcinomas, SCLC [27, 29,
<i>-A3, -A6</i>				125] - sarcoma, NSCLC [27, 29] - thyroid medullary carcinoma, H/N tumors, bronchial SCC [125] - laryngeal tumors [29] - leukemias [27]
<i>MAGE-A1</i>	B53	DPARYEFLW	Chaux et al., 1999a [16]	Melanoma, breast carcinoma, SCLC [27, 29, 125] -
<i>MAGE-A1</i>	Cw2	SAFPTTNF	Chaux et al., 1999a [16]	sarcoma, colon carcinoma, NSCLC [27, 29] - thyroid
<i>MAGE-A1</i>	Cw3	SAYGEPRKL	Chaux et al., 1999a [16]	medullary carcinoma [125]
<i>MAGE-A1</i>	Cw16	SAYGEPRKL	van der Bruggen et al., 1994b [127]	
<i>MAGE-A2</i>	A2	KMVLVHFL	Visseren et al., 1997 [128]	Melanoma, colon and breast carcinoma, SCLC [27, 29,
<i>MAGE-A2</i>	A2	YLQLVFGIEV	Visseren et al., 1997 [128]	124] - sarcoma, NSCLC [27, 29] - thyroid medullary

<i>MAGE-A2</i>	A24	EYLQLVFGI	Tahara <i>et al.</i> , 1999 [110]	carcinoma [125] - laryngeal tumors [77] - leukemias [27]
<i>MAGE-A3</i>	A1	EVDPIGHLY	Gaugler <i>et al.</i> , 1994 [40]	Melanoma, colon and breast carcinomas [27, 125] - H/N
<i>MAGE-A3</i>	A2	FLWGPRLV	van der Bruggen <i>et al.</i> , 1994a [126]	tumors [18] - bronchial SCC, thyroid medullary and
<i>MAGE-A3</i>	A24	TFPDLESEF	Oiso <i>et al.</i> , 1999 [89]	bladder carcinoma, sarcomas, SCLC, NSCLC [125] -
<i>MAGE-A3</i>	A24	IMPKAGLLI	Tanaka <i>et al.</i> , 1997 [111]	leukemias [29]
<i>MAGE-A3</i>	B44	MEVDPIGHLY	Herman <i>et al.</i> , 1996 [48], Fleischhauer <i>et al.</i> , 1996 [35]	
<i>MAGE-A3</i>	B52	WQYFFPVIF	Russo <i>et al.</i> 2000 [103]	
<i>MAGE-A4</i>	A2	GVYDGREHTV	Duffour <i>et al.</i> , 1999 [33]	Melanoma, NSCLC, sarcomas, esophageal, colon and breast carcinomas [27]
<i>MAGE-A6</i>	A34	MVKISGGPR	Zorn and Herend, 1999b [147]	Melanoma, NSCLC, colon carcinoma, leukemias [27]
<i>MAGE-A10</i>	A2	GLYDGMHL	Huang <i>et al.</i> , 1999 [52]	Not defined
<i>MAGE-A12</i>	Cw7	VRIGHLYIL	Paneli <i>et al.</i> , 2000 [91], Heidecker <i>et al.</i> , 2000 [47]	Melanoma, myeloma, brain tumors, sarcoma, leukemias, SCLC, NSCLC, H/N tumors, bladder, lung, esophageal, breast, prostate and colorectal carcinoma [27]
<i>BAGE</i>	Cw16	AARAVFLAL	Boël <i>et al.</i> , 1995 [6]	Melanoma, bladder and mammary carcinomas, H/N SCC, NSCLC, sarcoma
<i>DAM-6, -10</i>	A2	FLWGPRLV	Fleischhauer <i>et al.</i> , 1998 [36]	Melanoma, skin tumors, mammary and ovarian carcinomas

[77] - lung carcinoma [25, 77] - seminomas [25]					
GAGE-1, -2, -8	Cw6	YRPRRRY	Van den Eynde <i>et al.</i> , 1995 [123]	Melanoma, sarcoma, NSCLC, SCLC, mesothelioma,	
			De Backer <i>et al.</i> 1999 [26]	sarcoma, seminoma, leukemias, lymphomas, H/N tumors, bladder, esophageal, mammary, colon, prostate carcinomas	
GAGE-3, -4, -5, -6, -7B	A29	YYWPRRRY	De Backer <i>et al.</i> 1999 [26]	Melanomas, H/N tumors, leukemias, esophageal, lung and bladder carcinomas	
NA88-A	B13	MTQGQHFLQKV	Moreau-Aubry <i>et al.</i> , 2000 [82]	Melanoma	
NY-ESO-1	A2	SLLMWITQCFL	Jäger <i>et al.</i> , 1998 [54]	Melanoma, sarcoma, B-lymphomas, hepatoma, H/N	
	A2	SLLMWITQC	Jäger <i>et al.</i> , 1998 [54]	tumors, bladder, lung, prostate, ovarian, thyroid and	
	A2	QLSLLMWIT	Jäger <i>et al.</i> , 1998 [54]	breast carcinoma [18]	
NY-ESO-1a (CAG-3)	A31	ASGPGGGAPR	Wang <i>et al.</i> , 1998b [134]		

* Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope.

Table 2 Class I HLA-restricted melanocyte differentiation antigens. These antigens can only be expressed in normal and neoplastic cells of the same lineage (namely melanocytes, skin, retina, peripheral ganglia) or in normal cells of the prostate gland

<i>Gene</i>	<i>HLA allele</i>	<i>Peptide epitope</i>	<i>Authors [ref.]</i>
<i>MART-1/Melan-A^a</i>	A2	AAGIGILTV	Coulie <i>et al.</i> 1994 [22]
			Kawakami <i>et al.</i> , 1994a [58]
	A2	EAAGIGILTV	Schneider <i>et al.</i> , 1998 [106]
	A2	ILTVILGVL	Castelli <i>et al.</i> , 1995 [14]
	B45	AEEAAGIGIL	Schneider <i>et al.</i> , 1998 [106]
	B45	AEEAAGIGILT	Schneider <i>et al.</i> , 1998 [106]
<i>MC1R</i>	A2	TILGIFFL	Salazar-Onfray <i>et al.</i> , 1997 [104]
	A2	FLALIICNA	Salazar-Onfray <i>et al.</i> , 1997 [104]
<i>Gp100</i>	A2	KTWGQYWQV	Bakker <i>et al.</i> , 1995 [3]
	A2	AMLGTHTMEV	Tsai <i>et al.</i> , 1997 [120]
	A2	MLGTHTMEV	Tsai <i>et al.</i> , 1997 [120]
	A2	SLADTNSLAV	Tsai <i>et al.</i> , 1997 [120]
	A2	ITDQVPFSV	Kawakami <i>et al.</i> , 1995 [61]
	A2	LLDGTATLRL	Kawakami <i>et al.</i> , 1994b [59]
	A2	YLEPGPVTA	Cox <i>et al.</i> , 1994 [24]
	A2	VLYRYGSFSV	Kawakami <i>et al.</i> , 1995 [61]
	A2	RLMKQDFS	Kawakami <i>et al.</i> , 1998 [62]
	A2	RLPRIFCSC	Kawakami <i>et al.</i> , 1998 [62]
	A3	LIYRRRLMK	Kawakami <i>et al.</i> , 1998 [62]
	A3	ALNFPGSQK	Kawashima <i>et al.</i> , 1998 [65]
	A3	SLIYRRRLMK	Kawashima <i>et al.</i> , 1998 [65]

	A3	ALLAVGATK	Skipper <i>et al.</i> , 1996 [108]
	A24	VYFFLPDHL	Robbins <i>et al.</i> , 1997 [99]
	Cw8	SNDGPTLI	Castelli <i>et al.</i> , 1999 [15]
PSA	A1	VSHSFPHPLY	Corman <i>et al.</i> , 1998 [20]
	A2	FLTPKKLQCV	Correale <i>et al.</i> , 1997 [21]
	A2	VISNDVCAQV	Correale <i>et al.</i> , 1997 [21]
PSM	A1	HSTNGVTRIY	Corman <i>et al.</i> , 1998 [20]
Tyrosinase	A1	KCDICTDEY	Kittlesen <i>et al.</i> , 1998 [68]
	A1	SSDYVIPIGTY	Kawakami <i>et al.</i> , 1998 [62]
	A2	YMDGTMSQV	Wölfel <i>et al.</i> , 1994 [137]
	A2	MLLAVLYCL	Wölfel <i>et al.</i> , 1994 [137]
	A24	AFLPWHRLF	Kang <i>et al.</i> , 1995 [57]
	B44	SEIWRDIDF	Brichard <i>et al.</i> , 1996 [10]
TRP-1 (or gp75)	A31	MSLQRQFLR	Wang <i>et al.</i> , 1996b [132]
TRP-2	A2	SVYDFFVWL	Parkhurst <i>et al.</i> , 1998 [92]
	A2	TLDSQVMSL	Noppen <i>et al.</i> , 2000 [86]
	A31	LLGPGRPYR	Wang <i>et al.</i> , 1996a [131]
	A33	LLGPGRPYR	Wang <i>et al.</i> , 1998a [133]
	Cw8	ANDPIFVVL	Castelli <i>et al.</i> , 1999 [15]

^a Two different groups simultaneously discovered this gene and gave it two different names, MART-1 and Melan-A respectively.

Table 3 Class I HLA-restricted widely expressed antigens

Gene	HLA allele	Peptide epitope	Tissue distribution		Reference
			Tumors	Normal tissues	
ART-4	A24	AFLRHAAL	SCC, SCLC, H/N tumors, leukemia, lung,	Testis, placenta, fetal liver	Kawano <i>et al.</i> , 2000 [64]
		DYPSLSATDI	esophageal, gastric, cervical, endometrial,		
			ovarian and breast carcinomas		
CAMEL	A2	MLMAQEALAFI	Melanoma	Testis, placenta, heart, skeletal muscle,	Aamoudse <i>et al.</i> , 1999 [1]
				pancreas	
CEA	A2	YLSGANLNL	Melanoma	Testis, placenta, heart, skeletal muscle,	Tsang <i>et al.</i> , 1995 [121]
		(CAP-1)*		pancreas	
CEA	A3	HLFGYSWYK	Colon, rectum, pancreas, gastric, breast and lung carcinomas	Gastrointestinal embryonic tissue	Kawashima <i>et al.</i> , 1999 [66]
Cyp-B	A24	KFHRVIKDF	Lung adenocarcinoma, T cell leukemia,	Ubiquitously expressed in normal	Gomi <i>et al.</i> , 1999 [42]
		DFMIQGGDF	lymphosarcoma - bladder, ovarian, uterine and esophageal SCC	tissues.	
HER2/neu	A2	KIFGSLAFL	Melanoma - ovarian and breast carcinomas	Epithelial cells	Fisk <i>et al.</i> , 1995 [34]
HER2/neu	A2	IISAVVGIL	Melanoma, ovarian, pancreatic [96] ^b and breast carcinomas	Epithelial cells	Peoples <i>et al.</i> , 1995 [95]

<i>HER2/neu</i>	A2	RLLQETELV	Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas	Epithelial cells	Kono <i>et al.</i> , 1998 [71]
<i>HER2/neu</i>	A2	VVLGVVFGI ILEHGAYSL YMIMVKCWMI	Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas	Epithelial cells	Rongcun <i>et al.</i> , 1999 [101]
<i>HER2/neu</i>	A3	VLRENTSPK	Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas	Epithelial cells	Kawashima <i>et al.</i> , 1999 [66]
<i>hTERT</i>	A2	ILAKFLHWL	Lung and ovarian carcinomas - multiple myeloma, melanoma, sarcoma, acute leukemias, non-Hodgkin's lymphomas	Hematopoietic stem cells and progenitors; germinal center cells; basal keratinocytes; gonadal cells; certain proliferating epithelial cells	Vonderheide <i>et al.</i> , 1999 [131]
<i>hTRT</i>	A2	ILAKFLHWL RLVDDFLLV	Lung, prostate and ovarian carcinomas, multiple myeloma, melanoma, sarcoma, acute leukemias, non-Hodgkin's lymphomas	Circulating B cells; germinal center B cells; thymocytes; CD34+ progenitor hemopoietic cells	Minev <i>et al.</i> , 2000 [81]
<i>iCE</i>	B7	SPRWWPTCL	RCC	Kidney, colon, small intestine, liver, heart, pituitary gland, adrenal gland, prostate, stomach	Ronsin <i>et al.</i> , 1999 [102]

<i>MUC1</i>	A11	STAPPAHGV	Breast and ovarian carcinomas, multiple myeloma, B-cell lymphoma	None ^d	Domenech <i>et al.</i> , 1995 [31]
<i>MUC1</i>	A2	STAPPVHNV	Breast and ovarian carcinoma, multiple myeloma, B-cell lymphoma	None ^d	Brossart <i>et al.</i> , 1999 [11]
<i>MUC2</i>	A2	LLNQLQVNL MLWGWAREHV	Ovary, pancreas and breast mucinous tumors, colon carcinoma of non-mucinous type	Colon, small intestine, bronchus, cervix and gall bladder	Böhm <i>et al.</i> , 1998 [7]
<i>PRAME</i>	A24	LYVDSLFFL	Melanoma, H/N and lung SCC, NSCLC [122], RCC, adenocarcinoma, sarcoma, leukemias [122]	Testis, endometrium, ovary, adrenals, kidney, brain, skin	Ikeda <i>et al.</i> , 1997 [53]
<i>P15</i>	A24	AYGLDFYIL	Melanoma	Testis, spleen, thymus, liver, kidney, adrenal tissue, lung tissue, retinal tissue	Robbins <i>et al.</i> , 1995 [97]
<i>RUI</i>	B51	VPYGSFKHV	Melanoma, renal and bladder carcinomas	Testis, kidney, heart, skin, brain, ovary, liver, lung, lymphocytes, thymus, fibroblasts	Morel <i>et al.</i> , 2000 [83]

<i>RU2</i>	B7	LPRWPPQL	Melanoma, sarcomas, leukemia - brain, esophageal and H/N tumors - renal, colon, thyroid, mammary, bladder, prostatic and lung carcinomas	Testis, kidney, liver, urinary bladder [124]	Van den Eynde <i>et al.</i> 1999
<i>SART-1</i>	A24	EYRGFTQDF	Esophageal, H/N and lung SCC - adenocarcinoma, uterine cancer	Testis, fetal liver	Kikuchi <i>et al.</i> , 1999 [67]
<i>SART-1</i>	A*2601	KGSGKMKTE	Esophageal, H/N and lung SCC, adenocarcinoma, uterine cancer	Testis, fetal liver	Shichijo <i>et al.</i> , 1998 [107]
<i>SART-3</i>	A24	VYDYNCHVDL AYIDFEMKI	H/N, esophageal and lung SCC, adenocarcinoma, leukemia, melanoma	Lymphoid cells, fibroblasts, testis, fetal liver	Yang <i>et al.</i> , 1999 [139]
<i>WT1</i>	A2	RMFPNAPYL	Gastric, colon, lung, breast, ovary, uterine, thyroid and hepatocellular carcinomas - leukemia (including AML, ALL and CML)	Kidney, ovary, testis, spleen	Oka <i>et al.</i> , 2000 [90]

^a CAP-1 is an alternative name of this peptide.

^b Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope.

^c Telomerase is expressed in most human tumors: those listed were shown to be susceptible to lysis by cytotoxic T lymphocytes.

^d All epithelial tissues express mucin like hyperglycosylated molecules.

Table 4 Class I HLA-restricted tumor specific antigens, including both unique (CDK-4, MUM-1, MUM-2, β -catenin, HLA-A2-R170I, ELF2m, myosin-m, caspase-8, KIAA0205, HSP70-2m) and shared (CAMEL, TRP-2/INT2, GnT-V, G 250) antigens

Gene	HLA allele	Peptide epitope	Tissue expression		Reference
			Tumors	Normal tissues	
AFP	A2	GVALQTMKQ	Hepatocellular carcinoma	Fetal liver	Butterfield <i>et al.</i> , 1999 [12]
β -catenin/m	A24	SYLDSGIHF	Melanoma	None	Robbins <i>et al.</i> 1996 [98]
Caspase-8/m	B35	FPDSWCYF	H/N tumors	None	Mandruzzato <i>et al.</i> , 1997 [78]
CDK-4/m	A2	ACDPHSGHFV	Melanoma	None	Wölfel <i>et al.</i> , 1995 [138]
ELF2M	A68	ETVSEQSNV	Lung SCC	None	Hogan <i>et al.</i> , 1998 [50]
GnT-V	A2	VLDPVFIRC(V)*	Melanoma, brain tumors, sarcoma	Breast and brain (low expression)	Guilloux <i>et al.</i> , 1996 [45]
G250	A2	HLSTAFARV	RCC, colon, ovarian and cervical carcinomas	None	Visser <i>et al.</i> , 1999 [129]
HLA-A*0201-R170I	A2	CVFWLRIYLENGK	RCC	None	Brändle <i>et al.</i> , 1996 [9]
HSP70-2M	A2	SLFEGIDY	RCC, melanoma, neuroblastoma	None	Gaudin <i>et al.</i> , 1999 [39]
HST-2	A31	YSWMDISCWI	Gastric signet cell carcinoma	None	Suzuki <i>et al.</i> , 1999 [109]
KIAA0205	B44*03	AEPIINQTV	Bladder cancer	None	Gueguen <i>et al.</i> , 1998 [44]

<i>MUM-1</i>	B44	EEKLIVVLF	Melanoma	None	Coulie <i>et al.</i> , 1995 [23]
<i>MUM-2</i>	B44	SELFERSGLDY	Melanoma	None	Chiari <i>et al.</i> , 1999 [19]
<i>MUM-2</i>	Cw6	FRSGLDSYV	Melanoma	None	Chiari <i>et al.</i> , 1999 [19]
<i>MUM-3</i>	A28	EAFIQPTR	Melanoma	None	Baurain <i>et al.</i> , 2000 [4]
<i>Myosin/m</i>	A3	KINKNPKYK	Melanoma	None	Zorn and Hercend, 1999a [146]
<i>RAGE</i>	B7	SPSSNRIRNT	Melanoma, sarcomas, mesotheliomas, H/N tumors, bladder, renal, colon and mammary carcinomas	Retina only	Gaugler <i>et al.</i> , 1996 [41]
<i>SART-2</i>	A24	DYSARWNEI	H/N and lung SCC, lung	None	Nakao <i>et al.</i> , 2000 [85]
		AYDFLYNYL	adenocarcinoma, RCC, melanoma,		
		SYTRLFLIL	brain tumors, esophageal and uterine cancers		
<i>TRP-2/INT2</i>	A68	EVISCKLIKRR	Melanoma	None	Lupetti <i>et al.</i> , 1998 [76]
<i>707-AP</i>	A2	RVAALARDA	Melanoma	None ^b	Morioka <i>et al.</i> , 1995 [84]

^a VL PDVFIRC(V) = nonamer and decamer peptides are both recognized by CTLs.

^b This antigen is not expressed in normal cells but, as the tissue of the testis was not tested, it will not become clear to which category the antigen may belong until more information is available.

Table 5 Class II HLA-restricted antigens

Gene	HLA-allele	Peptide epitope	Tissue expression		Reference
			Tumors	Normal tissues	
Epitopes from normal protein antigens					
<i>Annexin II</i>	DRB*0401	DVPKWISIMTERSVPH	Melanoma	Not done	Li <i>et al.</i> , 1998 [73]
<i>Gp100</i>	DRB1*0401	WNRQLYPEWTEAQRLD	Melanoma	Melanocytes	Li <i>et al.</i> , 1998 [73]
<i>MAGE-I, -2, -3, -6</i>	DRB*1301, DRB*1302	LLKYRAREPVTKAE	Melanoma, lung and breast carcinomas, H/N SCC	Testis, placenta	Chaux <i>et al.</i> , 1999a [16]
<i>MAGE-3</i>	DR*1101	TSYVKVLHHMVKISG	Melanoma, lung and breast carcinomas, H/N SCC	Testis, placenta	Manici <i>et al.</i> , 1999 [79]
<i>MAGE-3</i>	DRB*1301, DRB*1302	AELVHFLLKYRAR	Melanoma, lung and breast carcinomas, H/N SCC	Testis, placenta	Chaux <i>et al.</i> , 1999b [17]
<i>MART-1/Melan-A</i>	DRB1*0401	RNGYRALMDKSLHVGTCALTRR	Melanoma	Melanocytes	Zarour <i>et al.</i> , 2000 [144]
<i>MUC1</i>	DR3	PGSTAPPAHGVT	Breast and ovarian cancers, multiple myeloma, B-cell lymphoma	None*	Hiltbold <i>et al.</i> , 1998 [49]

<i>NY-ESO-1</i>	DRB4*0101	VLLKEFTVSG	Melanoma, B-lymphoma, hepatoma [18] ^b , sarcoma, H/N tumors, - bladder, lung, prostate, ovarian, thyroid and breast carcinomas	Testis	Zeng <i>et al.</i> , 2000 [145]
<i>NY-ESO-1</i>	DRB4*0101-0103	PLPVPGVLLKEFTVSGNI VLLKEFTVSGNLTIRLT AADHRQLQLSISCLQQL	B-lymphoma, melanoma, sarcoma, H/N tumors, hepatoma [18] - bladder, lung, prostate, ovarian, thyroid and breast carcinomas	Testis	Jäger <i>et al.</i> 2000 [55]
<i>PSA</i>	DR4	ILLGRMSLEMPEDTG SLFHPEDTGQVFQ QVFQVSHSFPHPLYD NDLMLRLRLSEPAELT KKLQCVQLHVISM GVLQGITSMGSEPCA	Prostate carcinoma	Prostate gland	Corman <i>et al.</i> , 1998 [20]

<i>Tyrosinase</i>	DRB1*0401	QNILLSNAPLGPQFP	Melanoma	Melanocytes	Topalian <i>et al.</i> , 1994 [117]
		DYSYLQSDPDPSFQD			Topalian <i>et al.</i> , 1996 [118]
		SYLQDSDPDPSFQD			
<i>Tyrosinase</i>	DRB1*1501	RHRPLQEVYPEANAPIGHNRE	Melanoma	Melanocytes	Kobayashi <i>et al.</i> , 1998a [69]
<i>Tyrosinase</i>	DRB1*0405	EIWRDIDFAHE	Melanoma	Melanocytes	Kobayashi <i>et al.</i> , 1998b [70]
Epitopes from mutated protein antigens					
<i>HPV-E7</i>	DR*0401,	LFMDTILSFVCPLC	Cervical carcinoma	None	Höhn <i>et al.</i> , 1999 [51]
	DR*0407	LFMDSLNFVCPWC			
<i>CDC27/m</i>	DRB1*0401	FSWAMDLDPKGA	Melanoma	None	Wang <i>et al.</i> , 1999a [135]
<i>TP1/m</i>	DRB1*0101	GELIGILNAAKVPAD	Melanoma	None	Pieper <i>et al.</i> , 1999 [96]

^a All epithelial tissues express highly glycosylated mucins whereas tumor cells often show hypoglycosylated mucins with a normal protein sequence.

^b Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope.

Table 6 Epitopes derived from fusion proteins (fusion proteins are never found in normal tissues)

Gene	HLA allele	Peptide epitope	Tissue distribution among tumors	Reference
HLA class I restricted epitopes				
<i>bcr-abl</i> ^{IT}	A2	FMVELVEGA	CML	Buzyn <i>et al.</i> , 1997 [13]
		KLSEQESLL		
		MLTNSCVKL		
<i>bcr-abl p210(b3a2)</i>	A2	SSKALQRPV	CML	Yotnda <i>et al.</i> , 1998a [141]
<i>bcr-abl (b3a2)</i>	A3	ATGFKQSSK	CML	Greco <i>et al.</i> , 1996 [43]
		KQSSKALQR		
<i>bcr-abl p210 (b3a2)</i>	A3, A11	HSATGFKQSSK	CML	Bocchia <i>et al.</i> , 1996 [5]
<i>bcr-abl p210(b3a2)</i>	A3	KQSSKALQR	CML	Norbury <i>et al.</i> , 2000 [87]
<i>bcr-abl p210(b3a2)</i>	B8	GFKQSSKAL	CML	Norbury <i>et al.</i> , 2000 [87]
<i>ETV6/AML</i>	A2	RIAECTLGM	ALL	Yotnda <i>et al.</i> , 1998b [142]
HLA class II restricted epitopes				
<i>bcr-abl p190 (e1a2)</i>	DRB1*1501	EGAFHGDAEALQRPVAS	ALL	Tanaka <i>et al.</i> , 2000 [112]
<i>bcr-abl p210 (b2a2)</i>	DRB5*0101	IPLTINKEEALQRPVAS	CML	ten Bosch <i>et al.</i> , 1999 [116]

<i>bcr-abl p210 (b3a2)</i>	DRB1*0401	ATGFKQSSKALQRPVAS	CML	ten Bosch <i>et al.</i> , 1996 [115]
<i>bcr-abl p210 (b3a2)</i>	DRB1*1501	ATGFKQSSKALQRPVAS	CML	ten Bosch <i>et al.</i> , 1995 [114]
<i>bcr-abl (b3a2)</i>	DRB1*0901	ATGFKQSSKALQRPVAS	CML	Yasukawa <i>et al.</i> , 1998 [140]
<i>bcr-abl (b3a2)</i>	DRB1*1101	LIVVIVHSATGFKQSSKALQRPVA	CML	Pawelec <i>et al.</i> , 1996 [93]
<i>bcr-abl (b3a2)</i>	DR11	IVHSATGFKQSSKALQRPVASFEP	CML	Bocchia <i>et al.</i> , 1996 [5]
<i>Dek-cain</i>	DRB4*0103	TMKQICKKEIRRLHQY	AML	Ohnishi <i>et al.</i> , 1999 [88]
<i>LDLR/FUT</i>	DRB1*0101	GGAPPVTWRRAPAPG	Melanoma	Wang <i>et al.</i> , 1999b [132]
		WRRAPAPGAKAMAPG		
<i>Pml/RARα</i>	DR11	NSNHVASGAGEAAIETQSSSSEIV [28]	APL	Gambacorti-Passerini <i>et al.</i> , 1993 [38]
<i>p190 minor bcr-abl (ela2)</i>	DRB1*1501	EGAFHGDAEALQRPVAS	AML	Tanaka <i>et al.</i> , 2000 [112]
<i>TEL/AML1</i>	DP5, DP17	IGRIAECILGMNPSR	AML	Yun <i>et al.</i> , 1999 [143]

^a These bcr-abl epitopes are not true fusion proteins generated-epitopes, because they derive from outside the bcr-abl junction.

Table 7 Frequency of epitopes recognized by a given HLA allele

<i>Antigen</i>	<i>No. of epitopes</i>	<i>HLA-A</i>	<i>HLA-B</i>	<i>HLA-C</i>
<i>MAGE-1, -2, -3, -4, -6, -10, -12</i>	24	13 (54%)	7 (29%)	4 (17%)
<i>GAGE-1, -2, -3, -4, -5, -6, -7B, -8</i>	8	5 (62.5%)	0	3 (37.5%)
<i>MART-1</i>	6	4 (67%)	2 (33%)	0
<i>Gp100</i>	12	11 (92%)	0	1 (8%)
<i>Tyrosinase</i>	6	5 (83%)	1 (17%)	0

TABLE 8
HCV Peptides Previously Determined to Be Recognized
in HCV-Exposed Patients

HCV peptide	Sequence	HLA restriction
Core 1-9	MSTNPKPQK	A11
1-9*	MSTNPKPQR	A11
27-36	CQIVGGVYLL	B60
35-44	YLLPRRGPRLL	A2
41-49	GPRLGVRAT	B7
43-51	RLGVRAIRK	A3
51-59	KTSESRQPR	A3
88-96	NEGLGWCAW	B44
88-96*	NECCGWCAW	B44
132-140	DLMGYIPLV	A2
169-177	LPGCSFSIF	B7
178-187	LLALLSCLTV	A2
E1 234-242	NASRCWVAM	B35
257-266	QLRRHIDLLV	A2
290-298	QLFTFSRR	A3
E2 401-411	SLLAPGAKQNV	A2
453-465	PERLSRPLTDFD	A2
453-465*	PERLASRPLTDF	A2
460-469	RPLTDFDQGW	B53
489-496	YPPKPCGI	B51
569-578	CVIGGAGNNT	B50
621-628	TINYTIEK	A11
632-641	RMVVGVEHR	A3
721-729	LLFLLADA	A2
723-731	FLLLADARV	A2
NS2 826-838	LMALTLSFYKRY	A29
827-834	MALTSPY	A29
838-846	YISWCLWWL	A23
NS3 1073-1081	CINGVCWTV	A2
1131-1139	YLVRHADV	A2
1169-1177	LLCPAGHAV	A2
1261-1270	TLGFGAYMSK	A11
1262-1270	LGFGAYMSK	A3
1265-1274	GAYMSKAHCV	A3
1287-1296	TGAFVTYSTY	A2
1287-1296*	TGSPITYSTY	A2
1391-1399	LIFCHSKKK	A3
1395-1403	HSKCKCDEL	B8
1406-1415	KLVALGINAV	A2
NS4 1585-1593	YLVAYQATV	A2
1611-1618	LIRLKPTL	B8
1636-1643	TLTHPVTK	A11
1661-1669	VLVGGVLAA	A2
1764-1772	HMWNFISGI	A2
1789-1797	SLMAFTAAV	A2
1807-1816	LLFNILCGWV	A2
1851-1859	ILAGYGAGV	A2
1858-1867	GVAGALVAFK	A3
1859-1867	VAGALVAFK	A3
1915-1923	WMNRLIAFA	A2
NS5 2218-2226	NHDSFDAEL	B38
2252-2260	ILDSFDPLV	A2
2267-2276	REISVPAEIL	B60
2510-2518	SLTPPHSAK	A3
2578-2587	RLVFPDLGV	A2
2588-2596	RVCEKMALY	A3
2629-2637	KSKKTPMGF	B57
2727-2735	GLQDCTMLV	A2
2794-2802	HDGAGKRVY	B38
2794-2804	HDGAGKRVYYL	B38
3003-3011	VGYLLPNR	A31

from Anthony *et al Clinical Immunol.*, vol. 103, pages 264-276 (2002).

Table 9 HIV-CTL peptide epitopes

Peptide sequence	Gene product	Position	Clade specificity	HLA restriction
GSEELRSLY	p17	71-79	B	A1
ISERILSTY	Rev	55-63	B	A1
ILKD/EPVHGV	Pol	476-484	A/B	A2
SLF/YNTVATL	p17	77-85	A/B	A2
TLNAWVKV/V	p24	150-159	A/B	A2
ALKHRAVEL				
AFHHVAREL	Nef	190-198	A/B	A2
KIRLRPGGK	p17	18-26	A,B,D	A3
RLRDLLLVTR	gp41	775-785	B	A3
S/AIFQSSMTK	Pol	325-333	A/B	A3, A11, A33
DLSHFLKEK	Nef	86-94	B	A3
QVPLRPMTYK	Nef	73-82	B	A11
AVDLSHFLK	Nef	84-92	B	A11
IYQEPFKNLK	Pol	508-516	B	A11
TLYCVHQRI	p17	84-92	B	A11
(R)YLR/KDQQLL	gp41	591-598	A/B	A24
LFCASDAKAY	gp120	53-62	B	A24
DSRLAFHHM	Nef	186-194	B	A24
RDYVDRFFKTL	p24	296-306	A	A24
VSFEPIPIHY	gp120	263-272	B	A29
DTVLEDINL	Pol	85-93	A	A*6802
ETAYFYIKL	Pol	744-752	A,B,D	A*6802
ITLWQRPLV	Pol	58-67	A,B,D	A74
IPRRIRQGL	gp41	848-856	A,B,D	B7
TPGPGV/IRYPL	Nef	128-137	B	B7
FPVTPQVPLR	Nef	68-77	B	B7
SPRTLNAWV	p24	148-156	B	B7
GPKVKQWPL	Pol	171-180	A,B,C,D	B8
YKDDQQLL	gp41	586-593	B	B8
GGKKKYRL	p17	24-31	A	B8
DRFF/WKTLRA	p24	298-306	A/B	B14
DLNMMMLNIV				
DLNTMLNVV	p24	183-191	A/B	B14
ERYLRDQQL	gp41	589-597	A	B14
RAEQASQEV	p24	305-313	B	B14
YPLTFGWCF/F	Nef	135-143	B/D	B18, B49
FRDYVDRFY/FK	p24	293-302	B,D/A,C	B18
KRWII/MGLNK	p24	263-272	B	B27
TAVPWNASW	gp41	606-614	B	B35
VPLRPMTY	Nef	75-82	B	B35
H/NPDMVYQY	Pol	342-350	A/B	B35
PIPIVGDY	p24	260-268	B	B35
IPLTEAEAL	Pol	447-455	B	B51
DPNPQEVWL	gp120	77-85	B	B51
LPCRIKQII	gp120	378-385	B	B51
AT/SQEVKNWM	p24	177-185	A/B	B53
DTINEEAAEW	p24	203-212	A	B53
QATQEVKNW	p24	308-316	A	B53
EVKNWMTETL	p24	313-322	A	B53
TSTLQEIGW	p24	235-243	A,B	B57/58
I/ISPRTLNAW	p24	147-155	A/B	B57/58
KAFSPVPMF	p24	153-164	B	B57/58
QAISPRTL	p24	145-152	B	Cw3
SFNCGGEFF	gp120	376-383	B	Cw4
KYRLKHLVW	p17	728-736	A	Cw4
QASGEVKNW	p24	176-184	B	Cw4

from Kaul *et al J. Clinical Invest.*, vol. 107, pages 1303-1310 (2001)

Table 10

HCV viral peptide antigens

Peptide sequence	HLA restriction
CVIGGAGNNT	B50
GPRLGVRAT	B7
GPRLGVRA	B7
WHYPPKPCGI	B51
YPPKPCGIVPA	B51
YPPKPCGI	B51
STNPKPQK	A11
MSTNPKPKKNK	A11

from Koziel *et al J. Virol.*, vol. 67, pages 7522-7532 (1993)

Table 11

HCV viral peptide antigens

Peptide sequence	HLA restriction
CVNGVCWTV	A2
KLVALGINAV	A2

from He *et al PNAS USA*, vol. 96, pages 5692-5697 (1999)